Składam serdeczne podziękowania
Pani prof. dr hab. Małgorzacie Majcher
za poświęcony czas, umożliwienie realizacji własnych pomysłów,
szeroko pojętą opiekę naukową oraz wiarę we mnie
na każdym etapie studiów doktoranckich.

Bardzo dziękuję
pracownikom Pracowni Badania Związków Lotnych i Aktywnych Sensomicznie
za przyjazną atmosferę oraz wsparcie i motywację,
które ułatwiały realizację pracy doktorskiej.

Dziękuję również
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a w szczególności prof. UPP dr hab. Kamili Myszce i dr inż. Piotrowi Kubiakowi
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oraz przyjazną atmosferę podczas realizacji badań.

Pragnę podziękować
Mężowi, Rodzicom, Siostrze i wszystkim najbliższym mi ludziom,
za niesłabnącą motywację i zrozumienie,
ułatwiające realizację badań i napisanie tej rozprawy doktorskiej.

Uniwersytet Przyrodniczy w Poznaniu

Wydział Nauk o Żywności i Żywieniu

mgr inż. Kamila Szudera-Kończal

Wykorzystanie pleśni *Galactomyces geotrichum* do otrzymywania kompozycji aromatycznych z maślanki i serwatki. Charakterystyka związków aktywnych zapachowo w oparciu o podejście sensomiczne.

Praca doktorska wykonana

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Spis treści:

WYKAZ UŻYWANYCH SKRÓTÓW

GCxGC/ToF-MS – dwuwymiarowa chromatografia gazowa ze spektrometrią mas z analizatorem czasu przelotu (ang. *comprehensive two-dimensional gas chromatography with time-of-flight mass spectrometry*)

GC-MS – chromatografia gazowa ze spektometrią mas (ang. *gas chromatography – mass spectrometry*)

GC-O – chromatografia gazowa – olfaktometria (ang. *gas chromatography – olfactometry*)

HS-SPME – mikroekstrakcja do fazy stałej z fazy nadpowierzchniowej (ang. *headspace solid phace microextraction*)

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

OAV – wartość aktywności aromatu (ang. *odor activity value*)

OT – próg wyczuwalności sensorycznej (ang. *odor threshold*)

SAFE – destylacja pod obniżonym ciśnieniem (ang. *solvent assisted flavor evaporation*)

SIDA – analiza ilościowa z zastosowaniem izotopomerów (ang. *stable isotope dillution assay*)

STRESZCZENIE

Zmiany nawyków i preferencji żywieniowych konsumentów w połączeniu ze znacznym postępem technologicznym i skróceniem procesów produkcji żywności kształtują rosnące zapotrzebowanie na aromaty spożywcze. Dodatki aromatyzujące żywność mają za zadanie zapewnić wystarczająco intensywny aromat produktom przetworzonym. Szczególnie istotne staje się opracowanie nowych technologii produkcji aromatów naturalnych, które odpowiadają na aktualne potrzeby konsumentów. Wśród technologii pozyskiwania naturalnych związków aromatycznych, należy zwrócić uwagę na procesy biotechnologiczne. Wyróżniają się one wysoką wydajnością, możliwością zagospodarowania produktów ubocznych przemysłu, niezależnością od czynników klimatycznych i socjopolitycznych oraz relatywnie niskimi kosztami. Przykładem mikroorganizmu o dużym potencjale w produkcji związków zapachowych jest grzyb pleśniowy *Galactomyces geotrichum*, odpowiadający za miodowo-różany aromat tradycyjnie otrzymywanego sera smażonego. Celem badań przeprowadzonych w ramach niniejszej rozprawy doktorskiej było opracowanie parametrów procesu biotechnologicznego do otrzymywania kompozycji aromatycznych z maślanki oraz serwatki słodkiej i kwaśnej przy wykorzystaniu pleśni *G. geotrichum* oraz charakterystyka związków aktywnych zapachowo powstających w procesie fermentacji. Przeprowadzone badania obejmowały analizę 39 szczepów *G. geotrichum* pod kątem potencjału produkcji związków o zapachu miodowo-różanym, optymalizację warunków hodowli na podłożach z maślanką i serwatką oraz szczegółową, opartą o podejście sensomiczne, charakterystykę składu powstałych kompozycji aromatycznych.

Przeprowadzone badania pozwoliły na uzyskanie podstawowej wiedzy na temat wpływu warunków hodowli takich jak pH, temperatura oraz wpływu rodzaju źródła węgla w postaci dwucukrów lub cukrów prostych takich jak: sacharoza, laktoza, glukoza, galaktoza i fruktoza na biosyntezę związków zapachowych przez pleśnie *G. geotrichum* na podłożach zawierających maślankę lub serwatkę. Ponadto zaobserwowano, że jednoczesne prowadzenie fermentacji serwatki kwaśnej z dodatkiem LAB oraz pleśni *G. geotrichum* wpływa na wzbogacenie profilu zapachowego ze szczególnym wzrostem intensywności aromatu maślanego. Wyniki przeprowadzonych badań dostarczają podstawowej wiedzy na temat aromatu produkowanego przez grzyby pleśniowe

G. geotrichum na podłożach wykorzystujących produkty uboczne przemysłu mleczarskiego oraz możliwości jego intensyfikacji. Otrzymane kompozycje aromatyczne dają możliwość poprawy aromatu produktów spożywczych nie tylko w przemyśle mleczarskim, ale również cukierniczym, piekarniczym, czy piwowarskim.

ABSTRACT

Changes in consumers' eating habits and preferences, combined with significant technological progress and shortening of food production processes, shape the growing demand for food flavorings. These food additives are designed to provide a sufficiently intense aroma to processed food products. It is particularly important to develop new technologies for the production of natural flavors that respond to the current needs of consumers. Among the technologies for obtaining natural aroma compounds, special attention should be paid to biotechnological processes. They are distinguished by high efficiency, the possibility of using industrial by-products, independence from climatic and sociopolitical factors, and relatively low costs. An example of a microorganism with a high aroma potential is *Galactomyces geotrichum*, responsible for the honey-rose aroma of traditionally obtained fried cottage cheese. Therefore, the aim of the study performed in the doctoral dissertation was to develop the parameters of the biotechnological process for obtaining aroma compositions from buttermilk and sweet and sour whey using the *G. geotrichum* mold and to characterize aroma-active compounds formed in the fermentation process. The research carried out included the analysis of 39 strains of *G. geotrichum* in terms of the potential for the production of compounds with a honey-rose aroma, optimization of culture conditions on media with buttermilk and whey, and detailed characteristics of aroma compositions using the sensomic approach.

The conducted research allowed to obtain basic knowledge about the influence of culture conditions, such as pH, temperature, and the influence of the type of carbon source in the form of disaccharides or monosaccharides, such as: sucrose, lactose, glucose, galactose and fructose, on the biosynthesis of aroma compounds by *G. geotrichum* molds on media containing buttermilk or whey. In addition, it was observed that the simultaneous fermentation of sour whey with the addition of LAB and the mold *G. geotrichum* resulted in the enrichment of the odor profile with a particular increase in the intensity of the buttery aroma. The results of the conducted research provide basic knowledge about the aroma produced by *G. geotrichum* on substrates using by-products of the dairy industry and the possibilities of its intensification. The obtained aroma compositions give the opportunity to improve the aroma of food products not only in the dairy industry, but also in the confectionery, baking and brewing industries.

WYKAZ PUBLIKACJI WCHODZĄCYCH W SKŁAD ROZPRAWY DOKTORSKIEJ

P I. **Szudera-Kończal K.**, Myszka K., Kubiak P., Majcher M. A. (2020). The Use of Sour and Sweet Whey in Producing Compositions with Pleasant Aromas Using the Mold *Galactomyces geotrichum*: Identification of Key Odorants. Journal of Agricultural and Food Chemistry, 68 (39), 10799–10807. https://doi.org/10.1021/acs.jafc.0c03979.
(**IF_{2020}: 5.027**, punkty MNiSW: 140)

P II. **Szudera-Kończal K.**, Myszka K., Kubiak P., Majcher M. A. (2021). Analysis of the Ability to Produce Pleasant Aromas on Sour Whey and Buttermilk By-Products by Mold *Galactomyces geotrichum*: Identification of Key Odorants. Molecules, 26 (20), 6239. https://doi.org/10.3390/ molecules26206239
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P III. **Szudera-Kończal K.**, Myszka K., Kubiak, P., Drabińska N., Majcher M. A. (2023). The Combined Effect of Lactic Acid Bacteria and *Galactomyces geotrichum* Fermentation on the Aroma Composition of Sour Whey. Molecules, 28 (11), 4308. https://doi.org/10.3390/ molecules28114308
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1. WSTĘP

1. 1. Naturalne substancje aromatyczne

Substancje aromatyczne są jedną z grup dodatków do żywności. W związku z wpływem na podstawowe cechy produktów spożywczych, rynek aromatów spożywczych stanowi ponad ćwierć wartości rynku wszystkich dodatków do żywności (Longo i Sanromán, 2006). Zgodnie z danymi IMARC (Imarc Group, 2023), wartość ogólnoświatowego rynku substancji aromatyzujących żywność w 2021 roku wyniosła 15,6 miliardów dolarów, z prognozowanym wzrostem o 5 miliardów dolarów w 2027 roku. Tendencja ta wynika ze zmian nawyków żywieniowych konsumentów oraz intensywnego rozwoju technologii produkcji żywności. Zmiany stylu życia i nawyków żywieniowych konsumentów generują większe zapotrzebowanie na żywność funkcjonalną oraz żywność gotową do spożycia lub łatwą w przygotowaniu. Ponadto rosnąca świadomość konsumencka wpływa na wzrost znaczenia zrównoważonej produkcji i konsumpcji żywności oraz wysokiej jakość produktów spożywczych. Na znaczeniu zyskuje tzw. nowa żywność, która odpowiada na aktualne potrzeby konsumentów. Grupa ta obejmuje produkty nowo opracowane, innowacyjne, wyprodukowane dzięki nowatorskim technologiom lub procesom produkcyjnym (Czernyszewicz *i in.*, 2022). Niekonwencjonalne technologie produkcji żywności i skrócenie czasu jej produkcji mogą skutkować niedostatecznym rozwinięciem aromatu żywności lub brakiem aromatu charakterystycznego dla danego produktu. Przykładem takiej sytuacji jest proces produkcji substytutów mięsa na bazie białek roślinnych, które samodzielnie nie posiadają aromatu typowego dla mięsa. Kolejnym wyzwaniem dla dziedziny technologii żywności jest mięso hodowane komórkowo. W ostatnich latach prowadzonych jest coraz więcej badań na poziomie start-upów, które mają za zadanie stworzenie technologii umożliwiających produkcję mięsa hodowanego komórkowo i wprowadzenie go na rynek. Z uwagi na specyfikę procesu, taka forma mięsa jest jednak całkowicie pozbawiona aromatu i niezbędne jest jego odtworzenie (Kadim *i in.*, 2015). Opisane zmiany na rynku produkcji żywności generują konieczność stosowania większych ilości dodatków do żywności, mających za zadanie nadanie produktom spożywczym odpowiedniego aromatu. Innym rozwiązaniem, szczególnie istotnym w przemyśle fermentacyjnym, może być modyfikacja technologii produkcji w celu

zintensyfikowania aromatu, chociażby poprzez zastosowanie nowych, bardziej wydajnych szczepów mikroorganizmów.

Substancje aromatyczne stosowane jako dodatki do żywności dzielą się na trzy grupy: syntetyczne substancje aromatyczne, substancje aromatyczne identyczne z naturalnymi oraz naturalne substancje aromatyczne (Pijanowski *i in.*, 2004). Z uwagi na rosnącą świadomość żywieniową konsumentów, obserwuje się wciąż rosnący popyt na naturalne substancje aromatyczne w roli dodatków do żywności, kosztem syntetycznych substancji aromatycznych. Substancje aromatyczne pochodzenia naturalnego pozyskuje się na drodze ekstrakcji ze źródeł naturalnych (np. roślin) lub na drodze biotechnologicznej. Procesy te są przyjazne dla środowiska i projektowane w duchu zrównoważonego rozwoju, co nawiązuje do aktualnych przekonań społecznych (Sales *i in.*, 2018). Ekstrakcja aromatów ze źródeł naturalnych jest stosowana w przemyśle od dawna, jednak wiążą się z pewnymi ograniczeniami. Można wyróżnić wśród nich zależność od wielu czynników zewnętrznych: warunków pogodowych, chorób roślin i szkodników, sezonowości oraz wpływu sytuacji politycznej na dostępność surowców. Ponadto stosunkowo niskie stężenia związków aromatycznych w surowcach naturalnych sprawiają, że tego typu technologie są czasochłonne, skomplikowane i kosztowne. W obliczu tych wyzwań, szczególną uwagę zwraca się na opracowanie nowych metod biotechnologicznej produkcji substancji aromatycznych (Bicas *i in.*, 2010).

1. 2. Produkcja związków aromatycznych na drodze biotechnologicznej

Wśród biotechnologicznej produkcji związków aromatycznych wyróżnia się dwa rodzaje procesów. Pierwszym z nich jest synteza *de novo* obejmująca powstawanie złożonych związków aromatycznych z prostych cząsteczek budulcowych podczas szlaków metabolicznych mikroorganizmów. Drugim rodzajem jest biotransformacja (inaczej biokonwersja) prekursorów w jedno- lub kilkuetapowych reakcjach przez enzymy lub całe komórki mikroorganizmów (Sales *i in.*, 2018). Biotechnologiczne procesy produkcji aromatów są przyjazne dla środowiska i prowadzą do otrzymania produktów, które można nazywać naturalnymi. W odróżnieniu od ekstrakcji substancji aromatycznych z naturalnych źródeł, bioprocesy charakteryzują się wysokim stopniem kontroli, małym stopniem zależności od czynników zewnętrznych (np. sezonowości i zmian klimatu), wysoką wydajnością oraz stosunkowo niskim kosztem produkcji.

Procesy biokonwersji dają możliwość zastosowania produktów ubocznych przemysłu do produkcji związków aromatycznych, co jest podejściem nie tylko ekologicznym, ale również dodatkowo obniżającym koszty produkcji (Paulino *i in.*, 2020).

Na aromat produktów spożywczych składa się szereg związków aromatycznych. Percepcja aromatu żywności jako całość nie jest przy tym sumą aromatu pojedynczych związków wchodzących w jego skład. Literatura przedmiotu wskazuje, że mieszaniny zawierające już powyżej czterech związków aromatycznych tracą indywidualne nuty zapachowe na rzecz specyficznego, kompleksowego aromatu. Obecnie obserwuje się rosnące zainteresowanie technologiami produkcji kompozycji aromatycznych na drodze biotechnologicznej, które mogą lepiej odwzorować cechy danego produktu spożywczego, niż pojedyncze związki. Takie podejście eliminuje również konieczność wprowadzenia kosztownych i czasochłonnych procesów ekstrakcji wybranych związków do bioprocesów (Dunkel *i in.*, 2014).

1. 3. Charakterystyka grzyba pleśniowego *Galactomyces geotrichum*

Grzyby pleśniowe z rodzaju *Galactomyces* należą do królestwa *Fungi*, gromady *Ascomycota,* klasy *Saccharomycetes* i rodziny *Dipodascaceae* (de Hoog i Smith, 2004). Z uwagi na morfologiczny polimorfizm zależny od szczepu, który stoi na granicy między typowymi grzybami pleśniowymi i drożdżopodobnymi, rodzaj ten określa się jako nitkowate grzyby drożdżopodobne (Skóra *i in.*, 2009).

Grzyby *Galactomyces* spp. rozwijają się koncentrycznie na podłożu stałym, tworząc białe, filcowate kolonie. Na podłożach płynnych tworzą natomiast kożuch i/lub osad (Skóra *i in.*, 2009). Telemorfy *Galactomyces* wyróżniają się jednym z najkrótszych czasów generacji wśród eukariontów, który wynosi 1,1 h na podłożach płynnych w 30°C. Znacznie dłuższy jest czas generacji na podłożach stałych, który wynosi 3,6 h oraz faza spoczynkowa lag, wynosząca 10 h (Boutrou i Guéguen, 2005). Mikroorganizmy z rodzaju *Galactomyces* charakteryzują się zdolnością do wzrostu w środowisku tlenowym lub mikroaerofilnym oraz w szerokim zakresie temperatur (5-38°C) i pH (3,0-11,0) z optimum na poziomie 25-30°C i 5,0-7,0 (Skóra *i in.*, 2009).

Galactomyces spp. posiadają zdolność do asymilacji D-galaktozy D-galaktozy, L-sorbozy, fruktozy i soli kwasu octowego, przy czym poszczególne szczepy wykazują zdolność do asymilacji również innych źródeł węgla (Boutrou i Guéguen, 2005), jak kwas

mlekowy i mleczan. Grzyby *G. geotrichum* charakteryzują się również zdolnością proteolityczną i lipolityczną (Perkins *i in.*, 2020).

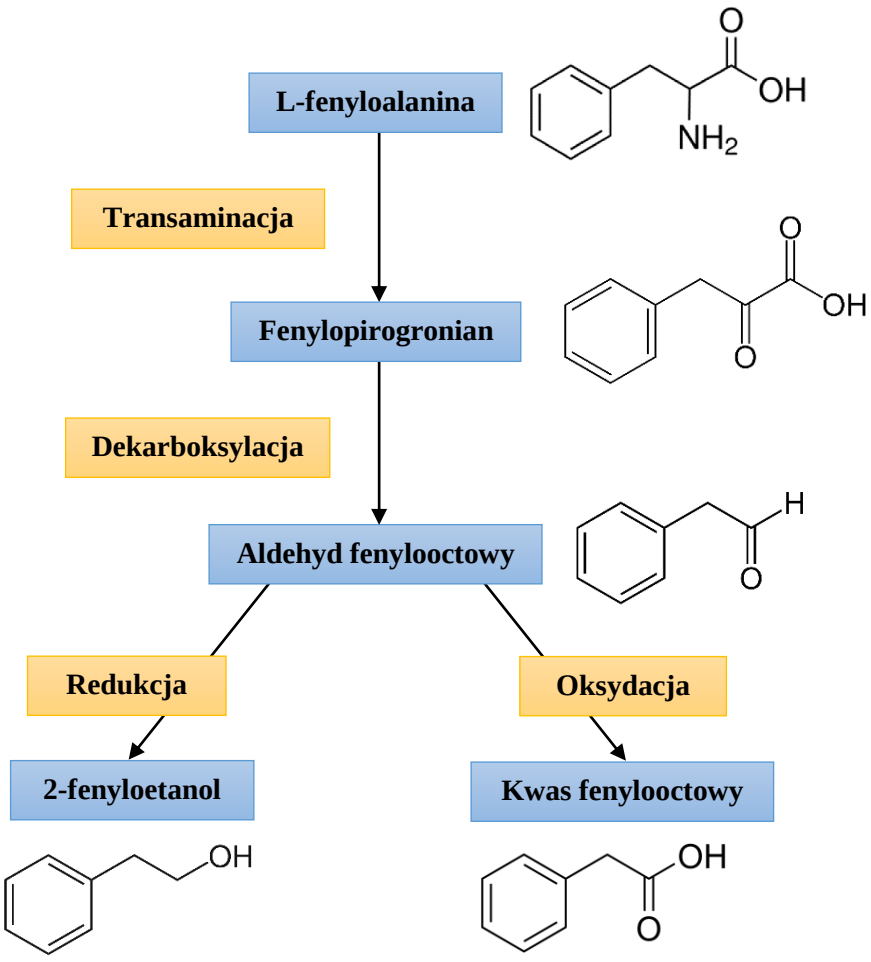
Grzyby pleśniowe z rodzaju *Galactomyces* są rozpowszechnione w środowisku, występując naturalnie w glebie i na powierzchniach roślin. Niektóre gatunki tych mikroorganizmów mogą rozwijać się również w przewodach pokarmowych owadów i ssaków (Boutrou i Guéguen, 2005). W ujęciu występowania w produktach spożywczych, grzyby *G. geotrichum* są najbardziej rozpowszechnione w przemyśle mleczarskim. Ich obecność stwierdza się m.in. w serze smażonym, raclette i Camembert, a także w wodnym kefirze (Wyder *i in.*, 1999; Marcellino *i in.*, 2001; Majcher *i in.*, 2014; Tang *i in.*, 2016). Aktywność proteolityczna i lipolityczna *G. geotrichum* wpływa na właściwości sensoryczne produktów mleczarskich (Perkins *i in.*, 2020). Działanie peptydaz produkowanych przez te mikroorganizmy redukuje cierpkość serów przez rozkład gorzkich peptydów, będących produktem rozkładu β-kazeiny np. przez *Penicillium camemberti* (Wyder *i in.*, 1999; Boutrou i Guéguen, 2005). Wykazano również, że *G. geotrichum* oddziałują na właściwości odżywcze produktów spożywczych. Zaobserwowano, że niektóre szczepy tego gatunku są zdolne do zewnątrzkomórkowej produkcji wielonienasyconych kwasów tłuszczowych (głównie n-3), dając możliwość poprawy jakości żywieniowej produktów spożywczych (Grygier *i in.*, 2020). Warto podkreślić, że obecność *G. geotrichum* w produktach spożywczych ogranicza rozwój niepożądanej mikroflory, np. *Listeria monocytogenes* i grzybów *Mucor* spp., wpływając tym samym bezpieczeństwo i trwałość żywności (Perkins *i in.*, 2020).

1. 4. Produkcja związków zapachowych przez grzyby *G. geotrichum*

Grzyby pleśniowe *G. geotrichum* odpowiadają za aromat przetworów mlecznych dzięki ich szerokiej aktywności enzymatycznej (Boutrou i Guéguen, 2005). Wykazano, że mikroorganizmy te są odpowiedzialne za tworzenie 2-fenyloetanolu, aldehydu fenylooctowego oraz kwasu fenylooctowego – związków o zapachu miodowo-różanym w tradycyjnie otrzymywanym wielkopolskim serze smażonym (Majcher *i in.*, 2014). Alkohol fenyloetylowy jest związkiem powstającym w szlaku Ehrlicha, podczas biotransformacji L-fenyloalaniny (Etschmann *i in.*, 2002). Według opracowania Dunkel *i in.*, 2014 związek ten odpowiada za aktywne kształtowanie aromatu prawie 23% produktów żywnościowych, a w szczególności produktów otrzymywanych na drodze

fermentacji, takich jak wino, piwo, sos sojowy czy chleb żytni. Dodatkowo 2-fenyloetanol jest szeroko stosowany jako substancja aromatyzująca w przemyśle spożywczym, perfumeryjnym i kosmetycznym, co generuje konieczność opracowywania kolejnych technologii jego pozyskiwania. Wśród tych metod szczególną uwagę zwraca się na procesy biotechnologiczne, umożliwiające otrzymanie alkoholu fenyloetylowego o pochodzeniu naturalnym.

Ze względu na specyfikę szlaku Ehrlicha, 2-fenyloetanol zwykle powstaje równocześnie z jego prekursorem aldehydem fenylooctowym oraz jego pochodną kwasem fenylooctowym. Wynika to z przekształcenia aminokwasu L-fenyloalaniny, na które składa się: transaminacja L-fenyloalaniny, dekarboksylacja powstałego fenylopirogronianu i redukcja otrzymanego aldehydu fenylooctowego do 2-fenyloetanolu oraz oksydacja do kwasu fenylooctowego (Etschmann *i in.*, 2002; Zhang *i in.*, 2017).



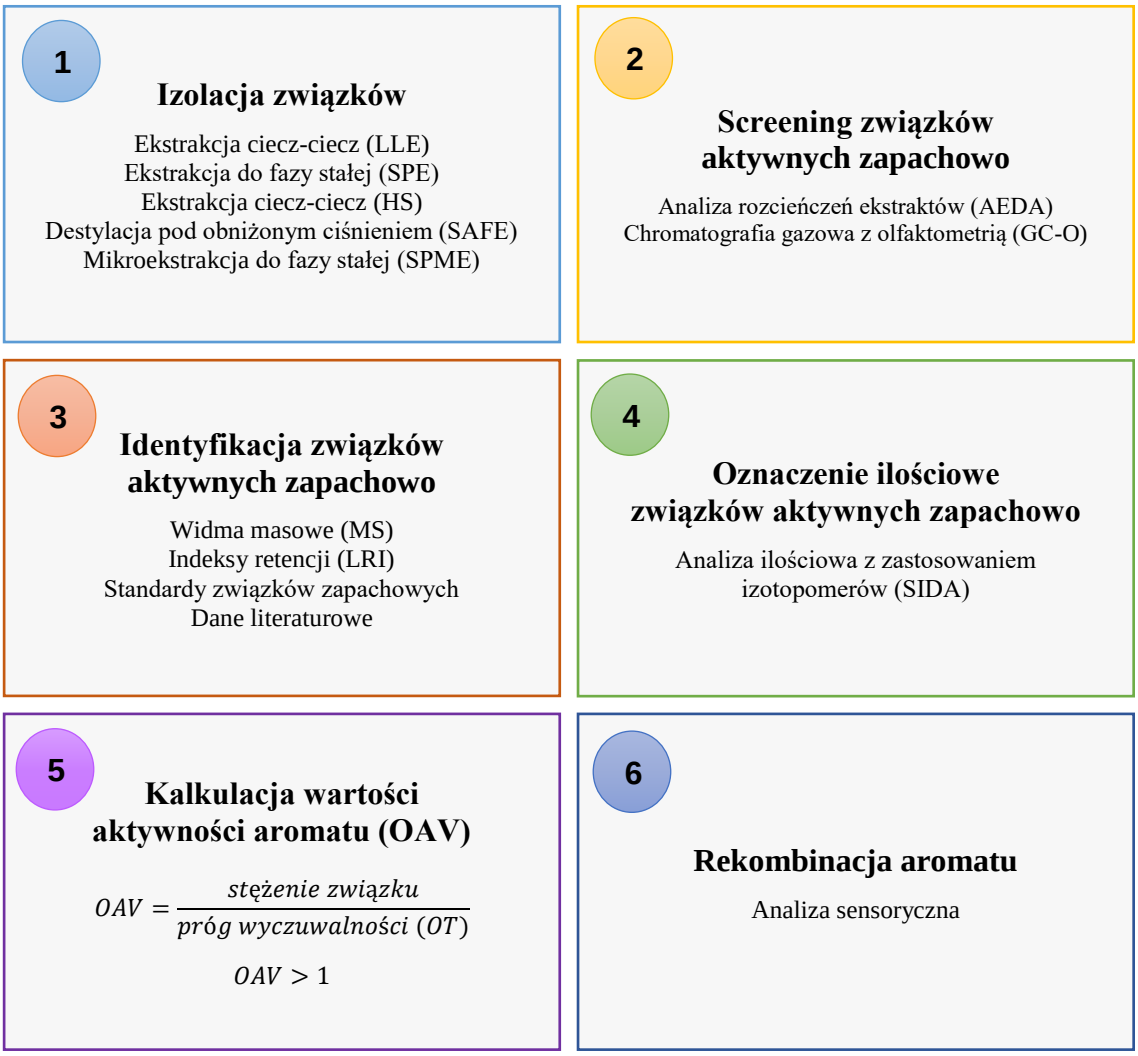
Rys. 1. Transformacja L-fenyloalaniny podczas szlaku Ehrlicha
[opracowanie własne na podstawie: Etschmann i in., 2002, Zhang i in., 2017].

Wszystkie te związki charakteryzują się zapachem określanym jako miodowo-różany i mogą przyczyniać się do aromatyzowania produktów spożywczych. Udział w kształtowaniu aromatu żywności przez te związki jest zależny od ich progu wyczuwalności sensorycznej (OT) w danej matrycy oraz stężenia, które jest zmienne w różnych produktach. Ponieważ każdy ze związków powstających w szlaku Ehrlicha cechuje inna wartość OT, ich stosunek ilościowy w produkcie spożywczym ma znaczny wpływ na końcowy aromat. W serze smażonym zaobserwowano niespotykany stosunek ilościowy aldehydu fenylooctowego o zapachu miodowym i 2-fenyloetanolu o zapachu różanym powstających w szlaku Ehrlicha, wynoszący blisko 1:1 (Majcher *i in.*, 2014). Obserwacja ta wskazuje na to, że grzyby *G. geotrichum* wyróżniają się wysokim potencjałem tworzenia przyjemnych kompozycji aromatycznych o intensywnym zapachu miodowo-różanym.

1. 5. Podejście sensomiczne w analizie związków zapachowych

Wprowadzenie chromatografii gazowej w latach sześćdziesiątych XX wieku zapoczątkowało rozwój badań dotyczących aromatu żywności. Przez lata zakładano, że całościowy aromat danego produktu jest wynikiem sumy intensywności nut zapachowych pojedynczych związków wchodzących w jego skład. Niestety próby odtworzenia aromatu żywności (np. oliwy z oliwek, soku pomarańczowego) na podstawie zidentyfikowanych związków zapachowych nie zawsze kończyły się sukcesem. Obserwacje te doprowadziły do opracowania podejścia sensomicznego w analizie zapachowej. Zakłada ono kompleksowe podejście do analizy aromatu żywności, które obejmuje koncentrację na identyfikacji kluczowych związków zapachowych danej kompozycji, a następnie ich precyzyjne oznaczenie ilościowe. Podejście sensomiczne opiera się na założeniu, że aromat jako całość jest nie tylko sumą nut zapachowych poszczególnych związków, ale specyficznym wrażeniem zależnym od składu kompozycji, stężeń kluczowych związków aromatu oraz składu matrycy produktu. Już brak jednego kluczowego związku lotnego lub jego nieprawidłowe stężenie może spowodować znaczne odchylenia w percepcji aromatu. Zastosowanie chromatografii gazowej i olfaktometrii (GC-O) w koncepcji sensomicznej umożliwia ograniczenie czasochłonnych procesów identyfikacji poszczególnych związków do substancji aktywnych zapachowo. Wykorzystanie izotopowo znakowanych standardów

zewnętrznych do oznaczeń ilościowych zidentyfikowanych związków w formie tzw. analizy rozcieńczeń stabilnych izotopów (SIDA) ogranicza ryzyko pominięcia związków śladowych lub otrzymania niedokładnych wyników, dzięki wysokiej precyzji. Z punktu widzenia sensomiki, istotnym czynnikiem wpływającym na aromat jest również matryca produktu, dlatego opracowano jednostkę wartości aktywności aromatu (OAV), zdefiniowanej jako stosunek stężenia związku zapachowego do jego progu wyczuwalności (OT) w danej matrycy. Analiza aromatu w ujęciu wartości OAV umożliwia precyzyjne wyznaczenie związków mających największy wpływ na całościowy aromat produktu spożywczego (Dunkel *i in.*, 2014).



Rys. 2. Schemat analizy aromatu z wykorzystaniem podejścia sensomicznego [opracowanie własne na podstawie: Dunkel i in., 2014].

2. CEL PRACY

Celem pracy doktorskiej było opracowanie parametrów procesu fermentacji do otrzymywania kompozycji aromatycznych z maślanki oraz serwatki słodkiej i kwaśnej przy wykorzystaniu pleśni *G. geotrichum* wraz z pełną charakterystyką profilu zapachowego oraz identyfikacją związków aktywnych zapachowo.

Zakres pracy obejmował następujące etapy:

1. Analizę 39 szczepów pleśni *G. geotrichum* w celu wyselekcjonowania szczepu zdolnego do produkcji nadmiaru aldehydu fenylooctowego w stosunku do 2-fenyloetanolu poprzez biotransformację L-fenyloalaniny w szlaku Ehrlicha.
2. Optymalizację procesu fermentacji poprzez dobór następujących parametrów: rodzaj cukru w pożywce, temperatura inkubacji, pH podłoża, dodatek LAB w kierunku uzyskania kompozycji aromatycznej o przyjemnym miodowo-różano-owocowym aromacie.
3. Określenie wpływu zastosowanego podłoża zawierającego serwatkę kwaśną, serwatkę słodką lub maślankę na profil sensoryczny oraz skład związków zapachowych tworzących kompozycję aromatyczną.
4. Identyfikację związków aktywnych zapachowo w otrzymanych kompozycjach aromatycznych przy wykorzystaniu podejścia sensomicznego, które obejmuje izolację związków zapachowych poprzez destylację pod obniżonym ciśnieniem (SAFE) i mikroekstrakcję do fazy stałej z fazy nadpowierzchniowej (HS-SPME), identyfikację kluczowych związków zapachowych z wykorzystaniem chromatografii gazowej i olfaktometrii (GC-O) i chromatografii gazowej ze spektrometrią mas (GC-MS), analizę ilościową z zastosowaniem izotopomerów (SIDA) oraz wyznaczenie wartości aktywności aromatu (OAV) jako stosunku stężenia związku do jego progu wyczuwalności sensorycznej.
5. Profilową analizę sensoryczną otrzymanych kompozycji aromatycznych.

3. HIPOTEZY BADAWCZE

Hipotezy badawcze badań przedstawionych w rozprawie doktorskiej były następujące:

1. Pleśnie *Galactomyces geotrichum* posiadają zdolność do biosyntezy związków zapachowych o przyjemnym miodowym, różanym i owocowym aromacie z półproduktów przemysłu mleczarskiego, takich jak maślanka i serwatka.

2. Istnieje możliwość sterowania jakością aromatu produktu uzyskanego na drodze fermentacji za pomocą składników pożywki np. rodzaju cukru oraz warunków hodowli np. pH, temperatura.

3. Zastosowanie podejścia sensomicznego pozwala na szczegółową identyfikację związków aktywnych zapachowo odpowiedzialnych za walory sensoryczne produktów fermentowanych.

4. METODYKA BADAŃ

4. 1. Materiał badawczy

Materiałem badawczym były produkty pofermentacyjne stanowiące podłoże bazowe bez lub z dodatkiem maślanki, serwatki słodkiej lub serwatki kwaśnej poddane fermentacji przez *G. geotrichum* lub kombinację *G. geotrichum* z bakteriami fermentacji mlekowej (LAB) w określonych warunkach i czasie.

4. 2. Metody analityczne

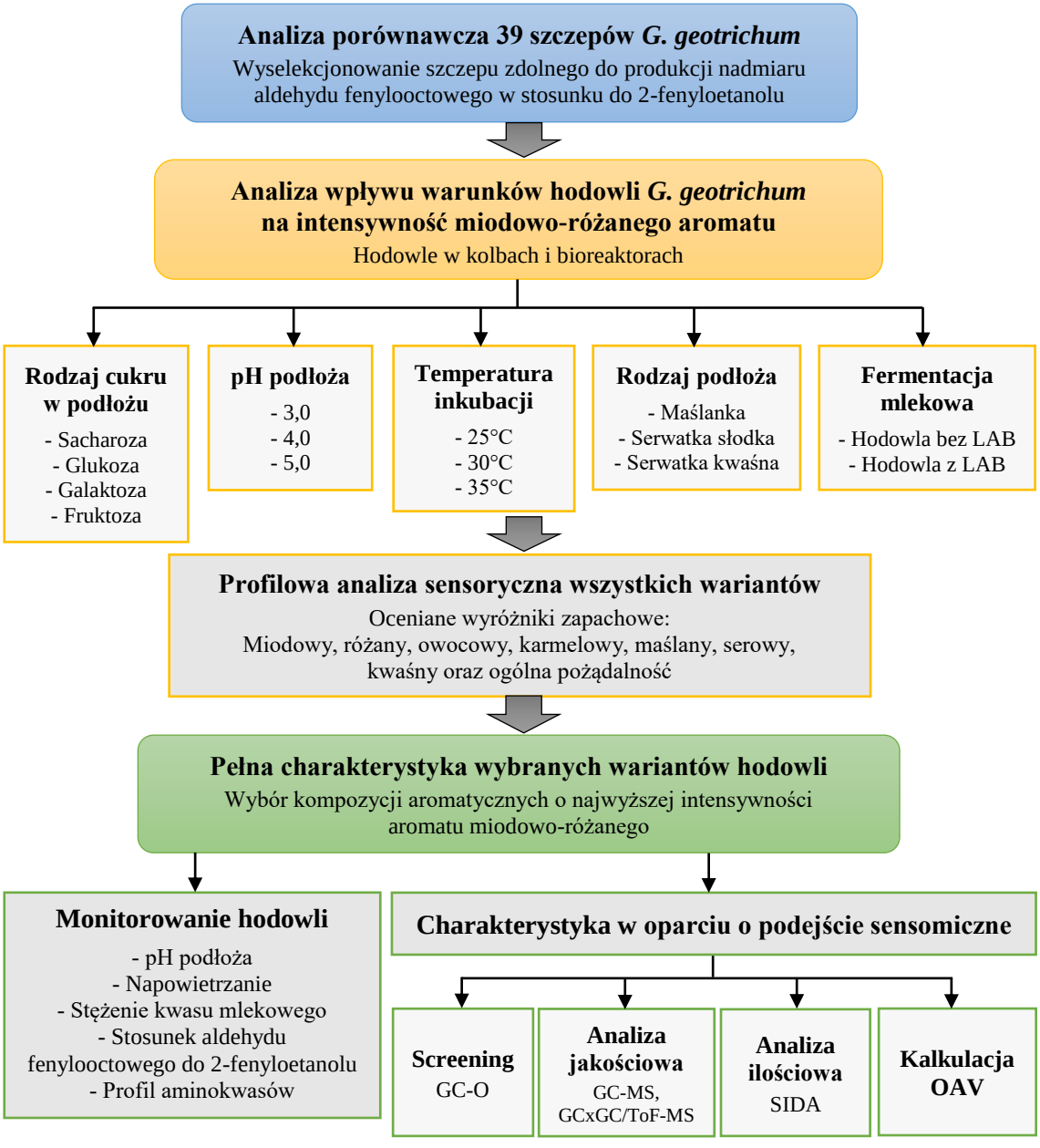
- 7-dniowe hodowle kolbowe przeprowadzono w kolbach stożkowych o pojemności 300 ml w łaźni wodnej z wytrząsaniem. **[P I, P II]**
- 7-dniowe i 8-dniowe hodowle w bioreaktorze w skali laboratoryjnej przeprowadzono w bioreaktorze Labfors 5 (Infors HT, Bottmingen, Szwajcaria) o pojemności 2,3 l. **[P I, P III]**
- Ocenę sensoryczną produktów pofermentacyjnych zrealizowano z zastosowaniem metody profilowej. **[P I-III]**
- Ekstrakcję aldehydu fenylooctowego i 2-fenyloetanolu z produktów pofermentacyjnych 39 szczepów *G. geotrichum* przeprowadzono z zastosowaniem metody HS-SPME. **[P I]**
- Ekstrakcję związków aktywnych zapachowo w produktach pofermentacyjnych przeprowadzono z zastosowaniem metody SAFE. **[P I-III]**
- Identyfikację związków aktywnych zapachowo w aromacie produktów pofermentacyjnych przeprowadzono z zastosowaniem metody GC-O. Dla wszystkich pików i deskryptorów aromatu z określonymi czasami retencji obliczono indeksy retencji na podstawie szeregu homologicznego n-alkanów C_7-C_{24}. **[P I-III]**
- Identyfikację i oznaczenie ilościowe analizowanych związków zapachowych w produktach pofermentacyjnych przeprowadzono z zastosowaniem metody GC-MS. Do oznaczenia ilościowego wykorzystano metodę SIDA. **[P I-III]**

- Oznaczenie ilościowe *G. geotrichum* podczas hodowli przeprowadzono przez przygotowanie rozcieńczeń dziesiętnych materiału badawczego i inkubację na płytkach Petriego z podłożem z chloramfenikolem. **[P II, P III]**
- Oznaczenie ilościowe LAB podczas hodowli przeprowadzono przez przygotowanie rozcieńczeń dziesiętnych materiału badawczego i inkubację na płytkach Petriego z podłożem MRS z agarem. **[P III]**
- Oznaczenie ilościowe kwasu mlekowego w podłożu z serwatką kwaśną po fermentacji przez LAB i *G. geotrichum* przeprowadzono z zastosowaniem wysokosprawnej chromatografii cieczowej (*ang. High-performance Liquid Chromatography*, HPLC). Kwas mlekowy zidentyfikowano na podstawie indeksu retencji. Oznaczenie ilościowe kwasu mlekowego przeprowadzono za pomocą zewnętrznej krzywej wzorcowej. **[P III]**
- Ekstrakcję i derywatyzację 11 wolnych aminokwasów w podłożu z serwatką kwaśną po fermentacji przez LAB i *G. geotrichum* przeprowadzono przy użyciu zestawu EZ:Faast™ do analizy wolnych (fizjologicznych) aminokwasów (Phenomenex, Aschaffenburg, Niemcy). Do analizy aminokwasów w produkcie pofermentacyjnym zastosowano chromatografię gazową sprzężoną ze spektrometrem masowym (GC-MS). Identyfikację i oznaczenie ilościowe poszczególnych aminokwasów przeprowadzono w odniesieniu do standardów zewnętrznych i znormalizowano w stosunku do standardu wewnętrznego. **[P III]**

Zastosowane metody analityczne zostały szczegółowo opisane w poszczególnych publikacjach wchodzących w skład pracy doktorskiej.

5. OMÓWIENIE WYNIKÓW

W pracy doktorskiej analizowano wpływ różnych warunków hodowli (rodzaj cukru w podłożu, pH podłoża, temperatura inkubacji) na intensywność aromatu wytwarzanego przez *G. geotrichum* na podłożach z maślanką oraz serwatką słodką i serwatką kwaśną. Badane warianty hodowli poddano dokładnej charakterystyce zarówno pod względem wyznaczenia profilu zapachowego za pomocą analizy sensorycznej jak i analizy instrumentalnej obejmującej identyfikację związków aktywnych zapachowo. Szczegółowy schemat przeprowadzonych doświadczeń znajduje się na rys. 3.



Rys. 3. Schemat doświadczeń zrealizowanych w pracy doktorskiej [opracowanie własne].

5. 1. Analiza 39 szczepów *G. geotrichum* w aspekcie produkcji aldehydu fenylooctowego i 2-fenyloetanolu

Pierwszym etapem przeprowadzonych badań była analiza 39 szczepów *G. geotrichum,* mająca na celu wyselekcjonowanie szczepu zdolnego do produkcji nadmiaru aldehydu fenylooctowego w stosunku do 2-fenyloetanolu poprzez biotransformację L-fenyloalaniny w szlaku Ehrlicha, co w efekcie miało zapewnić uzyskanie kompozycji aromatycznej o najwyższej intensywności miodowo-różanego zapachu. Badane szczepy zostały wyizolowane z wielkopolskiego sera smażonego, produkowanego w sposób tradycyjny, w którym odpowiadały za pojawienie się miodowych i kwiatowych nut zapachowych (Majcher *i in.*, 2014). Przeprowadzono 7-dniowe hodowle każdego szczepu w kolbach na podłożu podstawowym (Grygier *i in.*, 2015), zawierającym wodorofosforan sodu, kwas cytrynowy, chlorek magnezu, ekstrakt drożdżowy, sacharozę i L-fenyloalaninę, jako prekursor związków zapachowych o miodowo-różanym aromacie. Jako kryterium oceny intensywności aromatu miodowo-różanego produkowanego przez szczepy *G. geotrichum*, wybrano stosunek powierzchni pików aldehydu fenylooctowego i 2-fenyloetanolu.

Otrzymane wyniki wykazały, że każdy z analizowanych szczepów charakteryzował się zdolnością do produkcji aldehydu fenylooctowego i 2-fenyloetanolu na podłożu podstawowym. Wśród 39 szczepów *G. geotrichum,* dwa szczepy charakteryzowały się zdolnością do produkcji aldehydu fenylooctowego i 2-fenyloetanolu w stosunku bliskim 1:1, przy czym pozostałe szczepy produkowały znacznie więcej 2-fenyloetanolu. Najlepszym stosunkiem aldehydu fenylooctowego do 2-fenyloetanolu wyróżniał się szczep 32, dla którego wartość ta wyniosła odpowiednio 1.6:1. Obserwacja ta jest bardzo istotna z punktu widzenia produkcji aromatów na drodze biotechnologicznej ze względu na wysoką siłę aromatyzowania aldehydu fenylooctowego, który zwykle nie jest produkowany przez mikroorganizmy w wysokich stężeniach z uwagi na reakcje zachodzące podczas szlaku Ehrlicha. Próg wyczuwalności aldehydu fenylooctowego w wodzie wynosi 4 μg/kg, co jest wartością 60-krotnie niższą, niż próg wyczuwalności alkoholu fenyloetylowego w tych samych warunkach, który wynosi 240 μg/kg (Van Gemert, 2011). Dzięki temu zwiększenie udziału aldehydu fenylooctowego w kompozycji aromatycznej istotnie wpływa na zwiększenie intensywności odczucia aromatu **[P I]**.

Przeprowadzony eksperyment pozwolił na wyselekcjonowanie szczepu *G. geotrichum* z najwyższym potencjałem produkcji aldehydu fenylooctowego i 2-fenyloetanolu w stosunku ilościowym wyższym niż 1:1. Wyselekcjonowany szczep nr 32 był wykorzystywany w kolejnych badaniach.

5. 2. Optymalizacja warunków fermentacji podłoży z dodatkiem maślanki lub serwatki prowadzonej przez *G. geotrichum* w celu uzyskania kompozycji aromatycznej o intensywnym miodowo-różanym aromacie.

Warunki hodowli mają istotny wpływ na prowadzenie procesu fermentacji i co za tym idzie mogą być jednym z parametrów wykorzystywanych do kreowania bukietu finalnego produktu. Z tego względu w drugim etapie pracy doktorskiej zweryfikowano wpływ pH oraz temperatury hodowli na profil zapachowy uzyskanych kompozycji. Badania prowadzono w hodowlach kolbowych z wykorzystaniem wyselekcjonowanego szczepu *G. geotrichum,* który wyróżniał się zdolnością do produkcji nadmiaru aldehydu fenylooctowego w stosunku do 2-fenyloetanolu. Analizowano trzy warianty podłoży: z dodatkiem maślanki, serwatki słodkiej oraz serwatki kwaśnej. Produkty uboczne przemysłu mleczarskiego jako składniki podłoża częściowo odwzorowały naturalne środowisko rozwoju pleśni *G. geotrichum*, jakim jest wielkopolski ser smażony. Ponadto zawierają one L-fenyloalaninę, będącą prekursorem związków zapachowych o miodowo-różanym aromacie. Optymalizację warunków hodowli *G. geotrichum* poprzedzono badaniami wstępnymi, porównującymi ich zdolność do produkcji aldehydu fenylooctowego i 2-fenyloetanolu na podłożach z maślanką, serwatką słodką i serwatką kwaśną bez i z dodatkiem L-fenyloalaniny w różnych stężeniach (0,1%, 0,5%, 1,0% i 2,1%). Uzyskane wyniki wykazały, że wyselekcjonowany szczep *G. geotrichum* jest zdolny do produkcji tych związków w najwyższych stężeniach przy zawartości L-fenyloalaniny na poziomie 2,1% w każdym z badanych podłoży. Otrzymane kompozycje charakteryzowały się ponadto najlepszymi cechami sensorycznymi. Uzyskane wyniki wzięto pod uwagę w dalszych badaniach. Analiza sensoryczna wykazała, że prowadzenie fermentacji przy pH 5,0 prowadzi do otrzymania kompozycji o najszerszym profilu zapachowym, gdzie wyczuwano takie nuty zapachowe jak miodowe i owocowe w produkcie z serwatką kwaśną i słodką oraz miodowe i karmelowe w wariancie z maślanką. Warianty produktów pofermentacyjnych o początkowym pH

wynoszącym 3,0 i 4,0 charakteryzowały się znaczącym spadkiem intensywności każdego z analizowanych deskryptorów **[P I, II]**. W przypadku podłoża z maślanką, prowadzenie fermentacji przy niskim pH doprowadziło do uzyskania prawie bezzapachowego produktu **[P II]**. Wyniki analizy sensorycznej bioaromatów uzyskanych w różnych wariantach temperaturowych wykazały, że najbardziej optymalną temperaturą, skutkującą otrzymaniem kompozycji o najszerszym profilu sensorycznym jest 30°C. Otrzymane kompozycje aromatyczne charakteryzowały się wysoką intensywnością nut miodowych i różanych, a także owocowych w przypadku serwatki oraz karmelowych w przypadku maślanki. **[P I, II]**.

Najbardziej interesujące okazały się wyniki dotyczące wpływu źródła węgla w postaci dwucukrów lub cukrów prostych takich jak: sacharoza, glukoza, galaktoza i fruktoza na uzyskanie kompozycji aromatycznej przez pleśnie *G. geotrichum*. Należy zaznaczyć, że badania wstępne wykazały, że mikroorganizmy te nie metabolizują laktozy, dlatego nie została ona ujęta w badaniach. Uzyskane wyniki wskazują, że cukrem który najkorzystniej wpływał na uzyskanie pożądanego bioaromatu była galaktoza w przypadku podłoża zawierającego maślankę oraz sacharoza w przypadku podłoża zawierającego serwatkę słodką. Profil sensoryczny wariantu z serwatką kwaśną wyróżniał się największą intensywnością przy zastosowaniu galaktozy, jak również sacharozy jako źródeł węgla **[P I, II]**. Kompozycja zapachowa otrzymana po fermentacji podłoża z maślanką i galaktozą charakteryzowała się wysoką intensywnością aromatu miodowego. Zaobserwowano w tym wariancie również silną nutę karmelową, przy czym nuty różane i owocowe miały najmniejszy udział w całości kompozycji. Zastosowanie glukozy jako źródła węgla w podłożu z maślanką spowodowało zmniejszenie intensywności zapachu miodowego i karmelowego oraz obniżenie ogólnej pożądalności. Wyniki otrzymane dla wariantu z maślanką i fruktozą wykazały jeszcze większy spadek intensywności analizowanych wyróżników (z wyjątkiem nuty owocowej, gdzie nie zaobserwowano zmiany). W przypadku wariantu z maślanką i sacharozą zaobserwowano najmniej wyczuwalny aromat **[P II]**. Produkt otrzymany po fermentacji serwatki słodkiej z sacharozą charakteryzował się wysoką intensywnością wszystkich analizowanych wyróżników, a w szczególności aromatu miodowego. W tym przypadku zmiana źródła węgla na fruktozę lub glukozę spowodowała zmniejszenie intensywności zapachu różanego i karmelowego. Wariant z galaktozą charakteryzował się najwęższym profilem

zapachowym wśród wariantów zawierających serwatkę słodką **[P I]**. Analiza sensoryczna kompozycji otrzymanych po fermentacji podłoża z serwatką kwaśną wykazała, że galaktoza jako źródło węgla warunkowała nieco intensywniejszy aromat miodowy i różany otrzymanej kompozycji, a sacharoza – intensywniejszy aromat karmelowy i ogólną pożądalność. Obydwa warianty wyróżniały się silnymi, przyjemnymi nutami owocowymi. Fruktoza i glukoza jako składniki podłoża z serwatką kwaśną kolejno wpływały na zmniejszenie intensywności wszystkich analizowanych wyróżników **[P I, II]**.

Obserwacje powstałe podczas opisanego etapu optymalizacji warunków hodowli pleśni *G. geotrichum* wykorzystano w kolejnym etapie badań przeprowadzonych w skali bioreaktorowej. Na początku stwierdzono, że zwiększenie skali fermentacji wpłynęło na rozbudowanie bukietu zapachowego otrzymanej kompozycji co poskutkowało korektą wyróżników ocenianych w analizie sensorycznej, która polegała na dodaniu do niej wyróżników zapachu kwaśnego, serowego i maślanego. Wyniki analizy sensorycznej wykazały, że kompozycje aromatyczne powstałe po fermentacji podłoża z maślanką wyróżniają się wysoką intensywnością nut miodowych, maślanych i karmelowych **[P II]**. W przypadku serwatki zaobserwowano większą intensywność zapachu serowego i kwaśnego. Obserwacje te można powiązać z aromatem charakterystycznym dla analizowanych produktów ubocznych przemysłu mleczarskiego **[P I, II]**. Jednocześnie zauważono, że prowadzenie fermentacji podłoża z serwatką kwaśną i sacharozą w bioreaktorze laboratoryjnym wpływa na uzyskanie bioaromatu o najbardziej pożądanych cechach sensorycznych, w których dominowały takie nuty zapachowe jak miodowe, różane i owocowe **[P I]**.

Przedmiotem kolejnego etapu optymalizacji warunków hodowli była analiza wpływu wprowadzenia do hodowli LAB na intensywność miodowo-różanej kompozycji aromatycznej produkowanej przez *G. geotrichum* na podłożu z serwatką kwaśną. LAB są częścią naturalnego środowiska rozwoju pleśni *G. geotrichum*. Obecne są m.in. w wielkopolskim serze smażonym, z którego wyizolowano badane pleśnie (Majcher *i in.*, 2014). Warto zaznaczyć, że bakterie te są obecne również w serwatce kwaśnej, będącej produktem ubocznym powstającym podczas produkcji twarogu (Conde-Báez *i in.*, 2017). Ponadto LAB są zdolne do fermentacji laktozy, zapewniając tym samym dodatkowe źródło węgla *G. geotrichum,* niezdolnym do jej wykorzystania. Zaobserwowanie

najwyższej intensywności miodowo-różanego aromatu w wariancie hodowli z serwatką kwaśną i sacharozą pozwoliło założyć, że obecność metabolitów LAB w środowisku może wpływać na intensywność aromatu produkowanego przez *G. geotrichum*. W dostępnej literaturze badawczej opisano dotąd wpływ *G. geotrichum* na LAB (Chaves-López *i in.*, 2017), jednak nie analizowano dotąd odwrotnej zależności. Wprowadzenie LAB do procesu biokonwersji wpłynęło na wzbogacenie produkowanej kompozycji aromatycznej. Analizowana mieszanina zachowała intensywny aromat miodowy przy jednoczesnym wzroście wyczuwalności aromatu maślanego, karmelowego i owocowego. Ponadto wprowadzenie wstępnej fermentacji mlekowej do badanego bioprocesu zredukowało intensywność aromatu kwaśnego **[P III]**.

Optymalizacja warunków hodowli *G. geotrichum* na podłożach z maślanką i serwatką pozwoliła na dobór warunków i składników procesu skutkujących otrzymaniem aromatu o najwyższej intensywności przyjemnych nut miodowych, różanych, karmelowych i owocowych. Jako warunki fermentacji wybrano temperaturę 30°C oraz początkowe pH podłoża wynoszące 5,0. W aspekcie składu podłoża, wybrano warianty zawierające: serwatkę kwaśną i sacharozę, serwatkę kwaśną i galaktozę, serwatkę słodką i sacharozę oraz maślankę i galaktozę. Ostatnim wyselekcjonowanym wariantem hodowli była kombinacja pleśni *G. geotrichum* z LAB na podłożu z serwatką kwaśną i sacharozą.

5. 3. Charakterystyka aromatu produkowanego przez *G. geotrichum* na podłożach z maślanką i serwatką w oparciu o podejście sensomiczne

Dzięki zastosowaniu podejścia sensomicznego w charakterystyce kompozycji aromatycznych produkowanych przez *G. geotrichum*, nie tylko zidentyfikowano obecne w nich związki zapachowe, ale również określono ich udział w kształtowaniu aromatu. W tym celu obliczono dla każdego związku wartość aktywności aromatu (OAV), jako stosunek stężenia danego analitu do jego wartości OT (Pollner i Schieberle, 2016; Sahin i Schieberle, 2019). Za związki aktywne zapachowo uznano wszystkie, dla których wartość OAV>1.

W produktach pofermentacyjnych z serwatką słodką i serwatką kwaśną z sacharozą w formie źródła węgla zidentyfikowano dziesięć kluczowych związków zapachowych **[P I]**. Wprowadzenie do procesu dodatkowej fermentacji mlekowej pozwoliło na identyfikację dwunastu kluczowych odorantów w kompozycji powstałej po

biotransformacji podłoża z serwatką kwaśną i sacharozą **[P III]**. W kompozycjach aromatycznych powstałych na podłożach z maślanką i serwatką kwaśną z dodatkiem galaktozy zidentyfikowano trzynaście kluczowych związków zapachowych **[P II]**. We wszystkich analizowanych wariantach, związkami o najwyższych wartościach OAV były aldehyd fenylooctowy i/lub 2-fenyletanol, które odpowiadały za aromat miodowo-różany **[P I, P II, PIII]**.

Przeprowadzone badania wykazały, że w produktach pofermentacyjnych z serwatką słodką i kwaśną oraz sacharozą obecne są trzy związki o zapachu miodowo-różanym, powstające w szlaku Ehrlicha: aldehyd fenylooctowy, 2-fenyloetanol i kwas fenylooctowy. Zidentyfikowano je w stosunkach ilościowych wynoszących odpowiednio 1,7:1:0,2 w wariancie z serwatką kwaśną i 1,7:1:0,8 w wariancie z serwatką słodką. Należy przy tym zaznaczyć, że w przypadku serwatki słodkiej, stężenia aldehydu fenylooctowego i 2-fenyloetanolu były 1,7-krotnie niższe, a stężenie kwasu fenylooctowego 1,9-krotnie wyższe. Ze względu na najniższą wartość OT i najwyższe stężenie, aldehyd fenylooctowy ma największy wpływ na powstały aromat, czego dowodzi większa intensywność wyczuwanych organoleptycznie nut miodowych i różanych w kompozycji otrzymanej po fermentacji podłoża z serwatką kwaśną oraz wysoka wartość OAV (3010). W wielkopolskim serze smażonym, z którego wyizolowano analizowany szczep *G. geotrichum*, zaobserwowano występowanie aldehydu fenylooctowego, 2-fenyloetanolu i kwasu fenylooctowego w stosunku 0,7:1:0,2 przy stężeniu 2-fenyloetanolu na poziomie 1892 μg/kg po 4 dniach dojrzewania (Majcher *i in.*, 2014). Obecność tych związków obserwuje się również w innych produktach powstających na drodze fermentacji, jednak ich stężenia i stosunki ilościowe są bardzo zróżnicowane, wpływając tym samym na aromat produktu. Przykładem są fermentowane ziarna kakaowca, w których stwierdzono obecność miodowo-różanych związków zapachowych w stosunku 0,03:1:2,1 przy stężeniu 2-fenyloetanolu na poziomie 2100 μg/kg (Frauendorfer i Schieberle, 2019). W brzeczce poddanej fermentacji przez *Trametes versicolor* zidentyfikowano omawiane związki w stosunku 5,2:1:2,7 przy stężeniu 2-fenyloetanolu wynoszącym 31 μg/kg. Mimo najwyższej wartości OAV dla aldehydu fenylooctowego, aromat ten charakteryzował się jednak jedynie delikatnym, kwiatowym zapachem z uwagi na niskie stężenia wszystkich związków powstających w szlaku Ehrlicha (Zhang *i in.*, 2015). Produkty pofermentacyjne z serwatką słodką i

kwaśną oraz sacharozą wyróżniały się ponadto wysokimi wartościami OAV dla 3-(metylotio)-propanalu o zapachu gotowanych ziemniaków. Związek ten jest kluczowym odorantem ponad 53% produktów spożywczych, jednak w niewielu z nich można wyczuć za pomocą zmysłu węchu nutę gotowanych ziemniaków, co może oznaczać, że 3-(metylotio)-propanal wpływa na kształtowanie kompleksowego wrażenia zapachowego. Większość aromatów pochodzenia naturalnego, w tym kompozycja produkowana przez *G. geotrichum* to złożone mieszaniny różnych związków zapachowych. Wykazano, że kompozycje zawierające więcej niż cztery związki zapachowe, charakteryzują się utratą indywidualnych nut zapachowych niektórych związków, na rzecz kształtowania specyficznego aromatu jako całość (Dunkel *i in.*, 2014). W związku z tym, wysoka wartość OAV danego związku nie zawsze gwarantuje obecność charakterystycznej dla niego nuty zapachowej w aromacie. Przykładem takiej sytuacji jest olej rzepakowy, w którym w wyniku naturalnych przemian powstaje 2,3-butanedion, odpowiedzialny za charakterystyczny, maślany aromat. Wraz z nim produkowany jest jednak również trisiarczek dimetylu o aromacie kapusty oraz (E)-β-damascenon o zapachu gotowanych jabłek, przy czym ich wyróżniki, jako indywidualne nuty zapachowe, nie są wyczuwalne w produkcie. (Ruisinger i Schieberle, 2012).Wariant z serwatką kwaśną charakteryzował się również wysoką wartością OAV dla 3-metyl-1-butanolu o zapachu owocowym. Wynik ten bardzo dobrze koreluje z analizą sensoryczną, gdzie silnie wyczuwano nutę owocową w tym produkcie **[P I]**. Identyfikacja kluczowych odorantów w bioaromacie powstałym w wyniku fermentacji podłoża z serwatką kwaśną i sacharozą prowadzonej przez jednoczesne zastosowanie pleśni *G. geotrichum* i LAB wykazała ponad 20% spadek stężenia aldehydu fenylooctowego i 2-fenyloetanolu w produkcie pofermentacyjnym, w stosunku do fermentacji prowadzonej z zastosowaniem jedynie pleśni. Należy jednak podkreślić, że nie wpłynęło to na obniżenie intensywności odczuwania miodowych i różanych nut zapachowych, wręcz przeciwnie – odnotowano wzrost ich wyczuwalności. Można to wyjaśnić przez zachowanie wysokiego stosunku tych związków w połączeniu ze wzbogaceniem kompozycji przez blisko 8-krotnie wyższe stężenie kwasu fenylooctowego, niż w hodowli prowadzonej bez dodatku LAB. Stosunek stężeń aldehydu fenylooctowego, alkoholu fenyloetylowego i kwasu fenylooctowego w otrzymanej kompozycji aromatycznej wyniósł odpowiednio 1,7:1:2,5. Podobne

obserwacje wykazały badania Whetstine, Cadwallader i Drake, 2005, gdzie aldehyd fenylooctowy w połączeniu z kwasem fenylooctowym wpływał na zwiększenie intensywności aromatu sera Cheddar. Zaobserwowano również ponad 6-krotny wzrost stężenia 2,3-butanedionu oraz obecność 2,3-butanediolu, co wpłynęło na znaczny wzrost intensywności przyjemnego, maślanego aromatu. Obecność tych związków była niewątpliwie efektem fermentacji podłoża przez LAB. Związki o zapachu maślanym w dużej mierze kształtują aromat jogurtu i innych przetworów mlecznych poddawanych fermentacji mlekowej (Chen i Zhao, 2017). 2,3-butanedion powstaje podczas przemian cukrów i lipidów przy udziale bakterii i jest kluczowym związkiem zapachowym w różnych rodzajach serów, jak twaróg, Camambert, Lazur i Cheddar (Whetstine *i in.*, 2005; Deetae *i in.*, 2007; Majcher *i in.*, 2014, 2018). Drugi związek o zapachu maślanym – 2,3-butanediol jest natomiast zredukowaną formą acetoiny (również produkowanej przez LAB), będąc jednym ze związków kształtujących aromat tradycyjnego mleka fermentowanego „Lben" (Sarhir *i in.*, 2019). Ponadto wprowadzenie wstępnej fermentacji mlekowej do badanego bioprocesu zredukowało intensywność nieprzyjemnych nut zapachowych (octowych i serowych), dzięki redukcji stężenia kwasów. Otrzymane wyniki dostarczyły nowych, podstawowych informacji na temat jednoczesnego wpływu LAB i *G. geotrichum* na produkcję związków zapachowych **[P III]**.

W przypadku bioaromatu powstałego na podłożu z maślanką i galaktozą, najwyższą wartość OAV zaobserwowano dla aldehydu fenylooctowego. Związek ten, jako kluczowy odorant tej kompozycji, warunkował wystąpienie intensywnego aromatu miodowego. W produkcie tym zidentyfikowano również alkohol fenyloetylowy o zapachu różanym. Chociaż zaobserwowano niemal 1,3-krotnie wyższe stężenie tego związku, niż jego prekursora - aldehydu fenylooctowego, nie zaobserwowano wysokiej intensywności aromatu różanego w tym produkcie. Można to wyjaśnić 60-krotnie wyższą wartością OT 2-fenyloetanolu, a co za tym idzie – niższą wartość OAV. Istotną rolę w analizowanym bioaromacie odgrywał również 3-metylbutanal o aromacie słodowym. Kolejnym zidentyfikowanym związkiem o wysokiej wartości OAV był 2,3-butanedion, który warunkował wystąpienie intensywnych nut maślanych w kompozycji otrzymanej po fermentacji podłoża z maślanką i galaktozą **[P II]**.

Zastosowanie podejścia sensomicznego podczas charakterystyki otrzymanych kompozycji aromatycznych pozwoliło na identyfikację związków aktywnych zapachowo w poszczególnych produktach oraz określenie ich udziału w kształtowaniu aromatu. Szczegółowa identyfikacja kluczowych związków zapachowych jest niezbędna podczas oceny możliwego zastosowania kompozycji jako potencjalnego dodatku do żywności.

6. PODSUMOWANIE I WNIOSKI

Głównym osiągnięciem pracy doktorskiej jest uzyskanie podstawowej wiedzy na temat wpływu warunków hodowli takich jak pH, temperatura oraz wpływu rodzaju źródła węgla w postaci dwucukrów lub cukrów prostych takich jak: sacharoza, glukoza, galaktoza i fruktoza na biosyntezę związków zapachowych przez pleśnie *Galactomyces geotrichum* na podłożach zawierających maślankę lub serwatkę. W wyniku weryfikacji postawionych hipotez oraz na podstawie otrzymanych wyników badań, sformułowano następujące wnioski:

1. Grzyby *G. geotrichum* posiadają zdolność do biosyntezy związków zapachowych o przyjemnym miodowo-różanym aromacie z półproduktów przemysłu mleczarskiego w obecności L-fenyloalaniny. Profil zapachowy różni się w zależności od wykorzystania półproduktu. Kompozycje aromatyczne otrzymane po fermentacji serwatki kwaśnej charakteryzowały się wyższą intensywnością aromatu owocowego i kwaśnego, a w przypadku serwatki słodkiej – aromatu serowego. Aromat otrzymany po fermentacji podłoża z maślanką wyróżniał się wyższą intensywnością aromatu maślanego i karmelowego.
2. Optymalizacja warunków hodowli wyselekcjonowanego szczepu *G. geotrichum* na podłożach z maślanką, serwatką słodką oraz serwatką kwaśną pozwoliła na opracowanie parametrów hodowli umożliwiających otrzymanie kompozycji aromatycznej o intensywnym miodowo-różano-owocowym aromacie.
3. Kompozycja aromatyczna otrzymana po fermentacji podłoża z serwatką kwaśną i sacharozą charakteryzowała się najwyższą intensywnością przyjemnych, miodowo-różano-owocowych nut zapachowych.
4. Kluczowymi związkami zidentyfikowanymi w aromacie produkowanym przez *G. geotrichum* na podłożu z serwatką kwaśną i sacharozą są: aldehyd fenylooctowy, 3-metyl-1-butanol, 3-(metylotio)-propanal, 3-metylbutanal, trisiarczek dimetylu, 2,3-butanedion, 2-fenyloetanol, kwas butanowy, kwas octowy i kwas fenylooctowy.
5. Fermentacja serwatki kwaśnej przez *G. geotrichum* i LAB wpływa na zwiększenie intensywności miodowo-różanego aromatu przez jednoczesne

wytworzenie aldehydu fenylooctowego, 2-fenyloetanolu i kwasu fenylooctowego w stosunku ilościowym 1,7:1:2,5.

6. Kompozycja otrzymana przez fermentację podłoża z serwatką kwaśną przez *G. geotrichum* i LAB wyróżnia się 6,4-krotnie wyższą zawartością 2,3-butanedionu o zapachu maślanym, niż wariant bez dodatku LAB. W połączeniu ze zidentyfikowanym 2,3-butanediolem o tym samym zapachu, powstała kompozycja charakteryzuje się znacznie wyższą intensywnością aromatu maślanego, niż produkt po fermentacji wyłącznie przez *G. geotrichum*.
7. Zastosowanie podejścia sensomicznego pozwoliło na przeprowadzenie pełnej charakterystyki analizowanych kompozycji aromatycznych wraz z określeniem wpływu poszczególnych związków na kształtowany aromat.

Wyniki uzyskane w niniejszej pracy doktorskiej wpłynęły na rozwój dyscyplin jakimi są technologia żywności i żywienia oraz biotechnologia. Przeprowadzone badania podstawowe dają bezpośrednie rozwiązania na temat możliwości sterowania procesem fermentacji za pomocą składu podłoża czy też warunków hodowli w celu kształtowania aromatu produktów fermentowanych. Aromat otrzymanych produktów został poddany szczegółowej charakterystyce zarówno za pomocą analizy sensorycznej wskazując na preferencje odbierane przez ludzkie zmysły jak również za pomocą najnowszych technik instrumentalnych obejmujących identyfikację na poziomie molekularnym pojedynczych składników uzyskanego produktu.

7. LITERATURA

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OŚWIADCZENIA WSPÓŁAUTORÓW

Niniejszym oświadczam, że jestem współautorem publikacji:

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będącej częścią monotematycznego cyklu publikacji, stanowiącego moją rozprawę doktorską. Jednocześnie oświadczam, że publikacja powstała w wyniku indywidualnego wkładu pracy współautorów, określonego poniżej:

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Kamila Myszka – 5% – izolacja szczepów do badań, zabezpieczenie szczepu *G. geotrichum*, hodowla mikroorganizmów.
Piotr Kubiak – 5% – optymalizacja i kontrola hodowli bioreaktorowych mikroorganizmów.
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The Use of Sour and Sweet Whey in Producing Compositions with Pleasant Aromas Using the Mold *Galactomyces geotrichum*: Identification of Key Odorants

Kamila Szudera-Kończal, Kamila Myszka, Piotr Kubiak, and Małgorzata A. Majcher*

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ABSTRACT: Fermented products with a pleasant aroma and with strong honey, rose, and fruit odor notes were developed through the biotransformation of a medium containing sour or sweet whey with the addition of L-phenylalanine by the *Galactomyces geotrichum* mold. In order to obtain the strong honey-rose aroma, *G. geotrichum* strains were screened and fermentation conditions were optimized to achieve a preferable ratio (>1) of phenylacetaldehyde to 2-phenylethanol by the Ehrlich pathway. This allowed post-fermentation products with the ratio of concentrations of phenylacetaldehyde to 2-phenylethanol being 1.7:1. Additionally, the use of gas chromatography−olfactometry (GC−O) analysis and the calculation of odor activity values (OAVs) allowed 10 key odorants to be identified in post-fermentation products. The highest OAVs were found for phenylacetaldehyde with a honey odor in both sour and sweet whey cultures (3010 and 1776, respectively). In the variant with sour whey, the following compounds with the highest OAVs were 3-methyl-1-butanol (131), 3-(methylthio)-propanal (119), 3-methylbutanal (90), dimethyl trisulfide (71), 2,3-butanedione (37), and 2-phenylethanol (29). In the post-fermentation product with sweet whey, the following compounds with the highest OAVs were 3-(methylthio)-propanal (112), dimethyl trisulfide (69), and 2,3-butanedione (41).

KEYWORDS: *Galactomyces geotrichum, whey, fermentation, aroma-active compounds, GC−O, SIDA, OAV*

INTRODUCTION

There has, in recent years, been a growing demand for flavorings in industry, especially in food production.[1−3] These compounds enrich the aroma of food products, which is especially important given that modern food production technologies often simplify and shorten fermentation processes. Chemical synthesis of aroma compounds is highly efficient and results in a relatively inexpensive product, but these have low acceptability among consumers, who prefer foods containing additives of natural origin. This means that there is a need for relatively efficient and straightforward technologies that can produce volatile compounds of natural origin. Particular attention should be paid to biotechnological processes that do not suffer from the difficulties attendant on the extraction of aroma compounds from natural materials (including seasonality and effect of climate change on the acquisition of the raw materials), which can make use of industrial byproducts.[1,3−6]

Galactomyces geotrichum is a mold that occurs naturally in dairy products, which, according to our recent studies, is responsible for the formation of rose-like aroma compounds, such as phenylacetaldehyde, 2-phenylethanol, and phenylacetic acid.[7,8] We have, moreover, noted that the concentration ratio of phenylacetaldehyde and 2-phenylethanol is unusually close to 1:1 in the fried cottage cheese we tested. Phenylacetaldehyde has a lower odor threshold (OT) than 2-phenylethanol in a range of food matrices (60 times lower in water, 10 times lower in oil, and 4 times lower in starch),[7,9−11] which is why its highest content in the produced aroma is desirable.[6,7]

Whey is a yellowish liquid obtained during cheese production by separating the curd. About 160 million tons of whey is produced globally each year. The whey produced during cheese production is the most contaminated waste in this sector, which is why it is so important to develop new methods for managing it. During dairy production, sweet whey is obtained after enzymatic coagulation and sour whey comes from cottage cheese production. Depending on the type, whey contains 93−94% water, 4.5−6.0% lactose, 0.6−1.1% protein, 0.8−1.0% minerals, 0.05−0.9% lactic acid, and 0.06−0.5% fat.[12,13] It should be noted that whey contains L-phenylalanine, which is a precursor to the rose-like aroma compounds formed in the Ehrlich pathway.[14] In this study, both types of whey were analyzed, but sour whey is preferred to sweet on account of how it is produced: sour whey is formed during the production of cottage cheese when coagulation occurs by acidifying the milk to a pH of 5.1 or less. Fermentation by lactic acid bacteria results in more lactic acid than is produced in sweet whey, which affects the growth of *G. geotrichum*.[12,13,15] In addition, sour whey appears to be a more natural environment for the development of *G. geotrichum* from fried cottage cheese. During the production of this cheese, the

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environment is acidified by lactic acid bacteria, as with any kind of cottage cheese. Sour whey is formed in these types of processes, which is why it may also contain low-molecular-weight metabolites of lactic acid bacteria that can affect the metabolism of *G. geotrichum*. Furthermore, sour whey is more difficult to utilize than sweet whey, which is why we give special attention to new means of managing this byproduct.[16]

At present, there is increasing interest in the biotechnological production of aroma mixtures, rather than of individual aroma compounds,[17] as mixtures can more closely resemble the products of natural processes that occur during food fermentation.[3,17,18] In the present study, the final fermentation product has thus been treated as an aroma composition of several compounds, and all its key odorants have been identified, in order to find the compounds responsible not only for the honey-like and rose-like aroma notes but also for the fruit-like, caramel-like, or butter-like. Potential applications of this type of aroma are food products using fermentation processes such baking, brewing, and dairy industries. In this type of product, it is possible to utilize whey, and it is also desirable to enrich the aroma with honey-like and rose-like odor notes.[19]

The aim of this study was to determine the optimal conditions for the biotransformation by *G. geotrichum* of sour and sweet whey into post-fermentation products with an intense, honey-rose pleasant aroma. This is achieved by obtaining preferable ratio (>1) of phenylacetaldehyde to 2-phenylethanol through the Ehrlich pathway by screening of *G. geotrichum* strains and adjusting culture conditions. The post-fermentation product with the most intense, pleasant honey-rose-fruit aroma then underwent sensomics analysis,[17] which included identification of the key aroma compounds by gas chromatography−olfactometry (GC−O) and gas chromatography−mass spectrometry (GC−MS) and quantitation of the most important aroma compounds by the stable isotope dilution assay (SIDA) and by calculation of odor activity values (OAVs).

MATERIALS AND METHODS

Chemicals. Yeast extract, sucrose, glucose, and medium with chloramphenicol were obtained from BTL (Łódź, Poland). Galactose was purchased from Acros Organic (Geel, Belgium). Citric acid, $Na_2HPO_4{\cdot}2H_2O$, and $MgCl_2$ were obtained from POCH (Gliwice, Poland). L-Phenylalanine, lactic acid, sodium sulfate, dichloromethane, and diethyl ether were purchased from Sigma-Aldrich (Poznań, Poland). Inulin was obtained from Hortimex Plus (Konin, Poland). Spray-dried sour and sweet whey products were purchased from Laktopol (Suwałki, Poland). The following reference aroma compounds were purchased from Sigma-Aldrich (Poznań, Poland): 2,3-butanedione, acetic acid, 3-methylbutanal, 3-methyl-1-butanol, butanoic acid, 3-(methylthio)-propanal, dimethyl trisulfide, phenylacetaldehyde, 2-phenylethanol, and phenylacetic acid. The following stable isotopes were obtained from aromaLAB (Freising, Germany): [$^{13}C_4$] 2,3-butanedione, [$^{13}C_1$] acetic acid, [$^2H_3$] 2-methylbutanal, [$^2H_2$] 3-methyl-1-butanol, [$^2H_7$] butanoic acid, [$^2H_5$] 3-(methylthio)-propanal, [$^2H_6$] dimethyl trisulfide, [$^2H_5$] phenylacetaldehyde, [$^2H_5$] 2-phenylethanol, and [$^2H_8$] naphthalene.

Microorganisms. Thirty nine *G. geotrichum* strains were isolated during the ripening stage of Wielkopolski fried cheese produced in the vicinity of Poznań in Poland. The isolated strains were identified by amplification of the 18S rRNA coding sequence, lyophilized, and deposited in the microbial culture collection of the Department of Food Chemistry and Instrumental Analysis.

Screening of 39 *G. geotrichum* Strains. Shake flask cultivations were carried out in 300 mL Erlenmeyer flasks with 100 mL of medium containing per liter (modified, based on Grygier et al.[6]) 22.8 g of $Na_2HPO_4{\cdot}2H_2O$, 10.3 g of citric acid, 0.5 g of $MgCl_2$, and 0.17 g of yeast extract. After sterilizing the medium, 60 g of sucrose and 21 g of L-phenylalanine were added per liter; these had been exposed to UV radiation for 30 min before use. This procedure was intended to prevent the formation of aroma compounds due to high temperatures. This medium was employed in all the subsequent experiments. Each flask was inoculated with 10 μL of a *G. geotrichum* strain, which had previously been revived by inoculating the agar slant with chloramphenicol (incubation at 30 °C for 72 h under aerobic conditions). Fermentation in flasks with 39 strains of *G. geotrichum* was carried out in a water bath at 30 °C for 7 days with shaking (150 rpm).

Lyophilization of the *G. geotrichum* Strain. The strain selected during screening was lyophilized to standardize inoculum concentration. Four flasks with the selected mold strain were prepared in the same way as during screening. Fermentation in the flasks was carried out in a water bath at 30 °C for 72 h with shaking (150 rpm). The cultures were centrifuged for 10 min at 2012.4*g* (3000 rpm) and 20 °C after fermentation. The *G. geotrichum* precipitate was resuspended in 400 mL of the mixture of base medium and 10% inulin solution (1:1, v/v). To determine the number of colony-forming units (CFUs), the culture was sequentially diluted and plated onto agar plates with chloramphenicol. The plates were incubated for 72 h in 30 °C, and the CFUs were then counted to calculate the number of colonies per milliliter of sample. The freeze-drying process was conducted in a Beta 1-16 freeze dryer (Martin Christ, Osterode am Harz, Germany). The process was initiated with a freezing step at −35 °C for 2 h, followed by the main drying stage at 15 °C for 20 h, and the final drying at 22 °C (5 h). The dried preparations were collected into glass jars under a nitrogen atmosphere, each of which received the product formed by freeze-drying 3 mL of culture.

Optimization of Culture Conditions. We analyzed the effects of the type of sugar, the pH of the medium, and the incubation temperature on the sensory profile of the aroma produced by the selected strain of *G. geotrichum* mold. Optimization was carried out on two types of media: the first variant was prepared using the base medium with sour whey, while the second used sweet whey. In both cases, spray-dried whey was used at a concentration of 130 g/L. Citric acid as a component of the nutrient was replaced at this stage with lactic acid, as this naturally occurs in whey.[12] Once the medium had been prepared, the pH was adjusted to the set value in sterile conditions by adding lactic acid in the presence of litmus papers as pH indicators. Shake flask cultivations were carried out in 300 mL Erlenmeyer flasks with 200 mL of different medium variants. Each flask was inoculated with 3.4×10^6 CFU of lyophilized *G. geotrichum* mold.

Type of Sugar. We analyzed the effects of four types of sugar—sucrose, glucose, galactose, and fructose—on the aroma profile of the culture. Each type of sugar was added to the medium at a concentration of 60 g/L. As before, the UV-treated sugar was added to the sterilized medium. The pH of the media was adjusted to 5.0. Fermentation was carried out in a water bath at 30 °C for 7 days with shaking (150 rpm).

pH Value. The effect of pH on the culture was examined by carrying out fermentation on nutrient media with pH values of 3.0, 4.0, and 5.0. The type of sugar in the medium was sucrose. Fermentation was carried out in a water bath at 30 °C for 7 days with shaking (150 rpm).

Incubation Temperature. The incubation temperature was optimized by culturing *G. geotrichum* at three temperatures. The type of sugar in the medium was sucrose, and the pH value was 5.0. Incubation was carried out in water baths at different temperatures (25, 30, and 35 °C) for 7 days with shaking (150 rpm).

Sensory Evaluation of Cultures. Ten panelists experienced in descriptive sensory analysis carried out evaluation of the samples. The evaluation was run in triplicate in separate odor profiling sessions. The odor descriptors were selected from the basic flavor descriptive language (Givaudan Roure Flavor)[20] and were determined in preliminary tests, which looked for the variant with the highest

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possible ratio of phenylacetaldehyde to 2-phenylethanol. The qualities used were honey-like, caramel-like, rose-like, and fruit-like. The sensory panel also evaluated the general desirability of the aroma. Sensory analysis was performed by scoring odor descriptors on a 10 cm linear scale, with the beginning labeled "none" and the end labeled "very strong". We collected 20 g of *G. geotrichum* samples from the various culture condition variations and placed them in 100 mL glass containers. These were presented to the panelists at room temperature. The sensory evaluation of samples obtained from the bioreactor cultures was carried out in the same way except that, in addition to the previously mentioned odor descriptors, the following notes were also assessed: butter-like, cheese-like, and sour-like. The results were converted to numerical values for data analysis.

Bioreactor Cultures. For the biotechnological production of aroma composition, selected *G. geotrichum* mold culture parameters were used in a laboratory-scale bioreactor. Bioreactor cultures were carried out in a 2.3 L Labfors 5 bioreactor (Infors HT, Bottmingen, Switzerland) with a working volume of 2 L and aerated to 1.5 vvm, with the inlet air sterilized by filtration. The medium prepared in the same way as for previous flask cultures was stirred at a speed of 150 rpm. The batch fermentations were carried out in two variants, with sour whey and with sweet whey, and each variant was inoculated with 3.4×10^7 CFU of lyophilized *G. geotrichum* molds. The incubation temperature, medium pH (determined at the beginning of the culture), and type of sugar were determined in relation to the results obtained during the optimization of the culture parameters. The pH of the medium, as with the flask culture, was determined using lactic acid before inoculation and was not regulated during the experiment. Although the pH value was not actively controlled throughout the fermentation process, it was measured at the end with a value of 4.7. For each variant, also blank tests were performed with the same culture conditions, without inoculation of the medium with *G. geotrichum*.

Headspace Solid-Phase Microextraction (HS-SPME). HS-SPME was used as an isolation method to determine the levels of phenylacetaldehyde and 2-phenylethanol in samples collected during the screening of the 39 *G. geotrichum* strains. This employed divinylbenzene/Carboxen/polydimethylosiloxane (DVB/CAR/PDMS) fiber (Supelco, Bellefonte, PA, U.S.A.) in combination with an MPS 2XL multipurpose sampler (GERSTEL, Mülheim an der Ruhr, Germany). Samples (10 g) were placed in 20 mL headspace vials, spiked with internal standards (d_5-phenylacetaldehyde and d_5-2-phenylethanol) to a concentration of 500 ppb each, and sealed with septum magnetic caps. The compounds were extracted from headspace of the vials at 40 °C for 30 min. Afterward, the analytes were desorbed in the splitless mode at the GC injection port at 250 °C.

Solvent-Assisted Flavor Evaporation (SAFE). Odor-active compounds were isolated from the fermentation broth obtained by biotransformation of the sour and sweet whey in the bioreactor using the SAFE method described by Engel et al.[21] Samples (50 g) were mixed with dichloromethane (100 mL) and spiked with the internal standard [$^2H_8$] naphthalene (25 μg). The samples were then extracted for 2 h by shaking in the horizontal shaker, and the volatiles were isolated by SAFE extraction. In the next step, the extracts were dried over anhydrous sodium sulfate. Finally, the extracts were concentrated to approximately 500 μL using a Kuderna Danish concentrator (Sigma-Aldrich, Poznań, Poland).

GC−O. SAFE extracts underwent GC−O analysis to identify odor-active compounds, using an HP 5890 chromatograph (Hewlett-Packard, Wilmington, DE, U.S.A.) with two columns of different polarities: SPB-5 (30 m × 0.53 mm × 1.5 μm) and SUPELCOWAX 10 (30 m × 0.53 mm × 1 μm) (Supelco, Bellefonte, PA, U.S.A.). The effluent was divided between the olfactometry port with humidified air as a makeup gas and a flame ionization port using a Y splitter in GC. The operating conditions for the SPB-5 were as follows: an initial oven temperature of 40 °C (1 min) raised at 6 °C/min to 180 °C and at 20 °C/min to 280 °C. For SUPELCOWAX 10 column, the operating conditions were as follows: an initial oven temperature of 40 °C (2 min) raised to 240 °C at a 6 °C/min rate and held for 2 min isothermally. Injection of the SAFE extract (2 μL) into a GC column was using a splitless mode. GC-effluent sniffing (GC−O) was carried out by three panelists who detected odor-active regions and specified notes for the analyzed volatiles. For all peaks and flavor descriptors with specific retention times, retention indices were calculated in order to compare results with those obtained by GC−MS and literature data. For each compound, the retention indices (RIs) were calculated using a homologous series of C_7−C_{24} *n*-alkanes.

GC−MS. The analyzed chemical compounds were identified and quantified using a 7890A GC (Agilent Technologies, Santa Clara, CA, U.S.A.) coupled to a 5975C MSD (Agilent Technologies, Santa Clara, CA, U.S.A.).

GC−MS after SAFE Extraction. The apparatus was equipped with two columns: SUPELCOWAX 10 (30 m × 0.25 mm × 0.25 μm) and an SLB-5ms (30 m × 0.25 mm × 0.5 μm) (Supelco, Bellefonte, PA, U.S.A.). Helium was used as a carrier gas with a flow of 32.2 cm/s. The temperature programs were the same as for GC−O. Mass spectra were recorded in an electron impact mode (70 eV) in a scan range of *m/z* 33−350. Compounds were identified by comparing their mass spectra, RIs, and flavor notes on two columns of different polarities to those of standard compounds, National Institute of Standards and Technology (NIST) 09 Mass Spectral Library and literature data.

HS-SPME−GC−MS. This analysis was carried out using an HP-5ms column (30 m × 0.18 mm × 0.3 μm) (Hewlett-Packard, Wilmington, DE, U.S.A.) operating as follows: an initial oven temperature of 40 °C (1 min) raised at 9 °C/min to 200 °C and at 15 °C/min to 280 °C and held for 6 min isothermally. The other GC−MS conditions and the compound identification process were the same as for SAFE extract analysis.

Quantitation by Stable Isotope Dilution Assays (SIDA). All compounds identified during GC−O analysis were quantified by the SIDA method. To this end, stock internal standards of the labeled isotopes were prepared in diethyl ether and added to the samples of post-fermentation products after bioreactor culture, in concentrations similar to that present in the post-fermentation broth for each compound. The samples prepared in this way were extracted by the SAFE method. The identified aroma compounds were analyzed by GC−MS, monitoring the intensity of the respective ions presented in Table 1. For all volatiles, response factors were calculated in the standard mixture of labeled and unlabeled compounds at a known concentration of 500 ppb. In the case of 3-methylbutanal, for which

Table 1. Labeled Standards and Quantitation Ions Used for SIDA (Stable Isotope Dilution Assay) Concentration Calculations of 10 Key Odorants Present in the Post-Fermentation Product from the Bioreactor Culture

compound	quantitation ions (*m/z*)[a]	labeled standards	ion IS (*m/z*)[b]
2,3-butanedione	86	[$^{13}C_4$] 2,3-butanedione	90
acetic acid	60	[$^{13}C_1$] acetic acid	61
3-methylbutanal	86	[$^2H_3$] 2-methylbutanal	89
3-methyl-1-butanol	70	[$^2H_2$] 3-methyl-1-butanol	72
butanoic acid	73	[$^2H_7$] butanoic acid	77
3-(methylthio)-propanal	104	[$^2H_5$] 3-(methylthio)-propanal	107
dimethyl trisulfide	126	[$^2H_6$] dimethyl trisulfide	132
phenylacetaldehyde	120	[$^2H_5$] phenylacetaldehyde	125
2-phenylethanol	122	[$^2H_5$] 2-phenylethanol	127
phenylacetic acid	136	[$^2H_5$] 2-phenylethanol	127

[a]Ions of analytes used for quantitation. [b]Ions of internal standard IS (isotopologues) used for quantitation.

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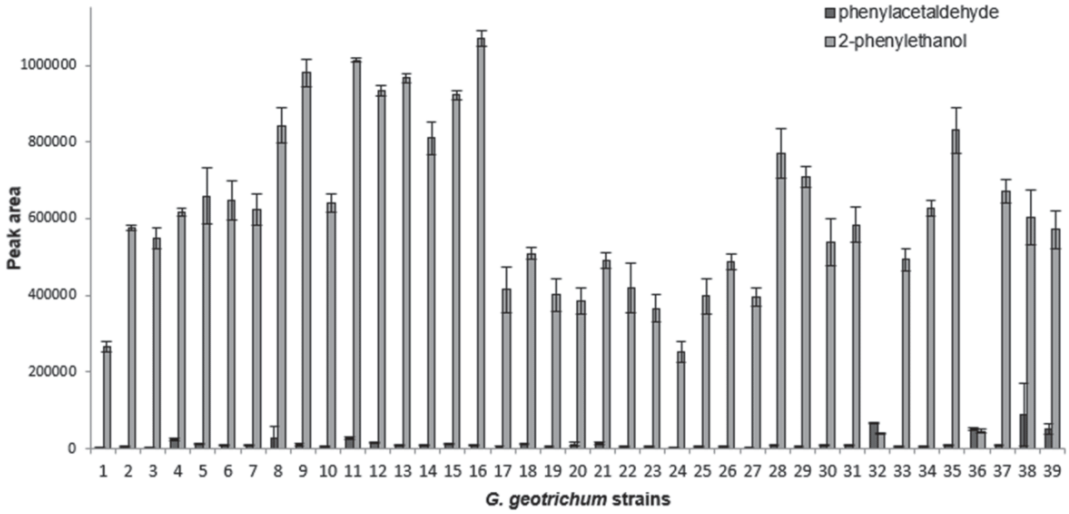

Figure 1. Peak areas of phenylacetaldehyde and 2-phenylethanol extracted from post-fermentation products obtained by biotransformation of the base medium by 39 strains of *G. geotrichum* by SPME.

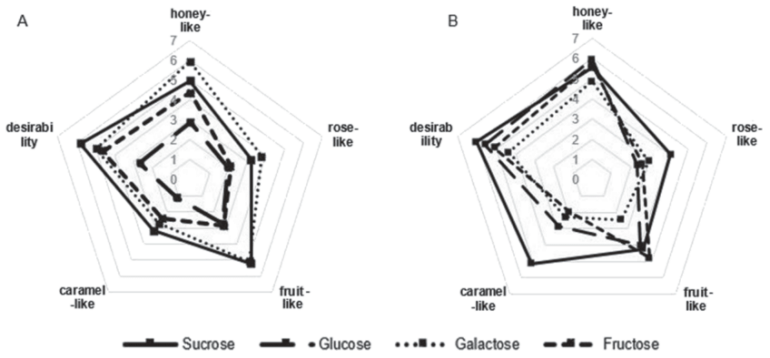

Figure 2. (A, B) Sensory profiles of post-fermentation products from flask cultures with (A) sour whey and (B) sweet whey for various types of sugars.

no direct isotopologue was available, we used a 2-methylbutanal isotopologue, which in our opinion is the most similar to this compound. We then applied the correction using the response factor. The concentrations of the analyzed volatiles in the samples were calculated using the peak area of the analyte and its corresponding internal labeled standard obtained for selected ions. The odor activity values (OAVs) were then calculated by dividing the concentration of a given analyte by its odor threshold (OT) value determined in water. The results obtained for the blank samples were subtracted from the results obtained for the sour and sweet whey products.

RESULTS AND DISCUSSION

Screening of *G. geotrichum* Strains. In the first experiment, 39 strains of *G. geotrichum* molds were characterized in terms of the ratio of phenylacetaldehyde to 2-phenylethanol. This experiment was intended to find those strains with the ability to produce more phenylacetaldehyde than 2-phenylethanol through the Ehrlich pathway. When L-phenylalanine is transformed, both compounds are usually produced and are present at the same time in the fermentation products; however, in most cases, 2-phenylethanol dominates in quantity over phenylacetaldehyde. Reversing the ratio to give higher amounts of phenylacetaldehyde would increase aromatization power. The results in Figure 1 thus illustrate the ratio of phenylacetaldehyde to 2-phenylethanol obtained during fermentation. It can be seen that most strains produced significantly greater amounts of 2-phenylethanol than phenylacetaldehyde. However, for strains 32 and 36, the ratios of phenylacetaldehyde to 2-phenylethanol were 1.6:1 and 1.1:1, respectively. Based on these results, strain 32 was selected for further study.

Optimizing Culture Conditions. The aim of this experiment was to optimize the culture conditions so as to increase the pleasant honey, rose-like aroma produced by the Ehrlich pathway from L-phenylalanine. To this end, the type of sugar added, the pH, and the incubation temperature were recorded. Each culture variant was subjected to a sensory analysis with four target odor descriptors: honey-like, rose-like, fruit-like, and caramel-like and general desirability.

The effect of the type of sugar in the medium on the sensory quality of the post-fermentation product is outlined in Figure 2. Our preliminary studies showed that *G. geotrichum* molds do not ferment lactose, which is why sucrose, glucose, galactose, and fructose were used in the experiment as nutrient ingredients. The results in Figure 2 illustrate that for both fermentation products, the panelists ranked general desirability highest when sucrose was used. In the sour whey culture, the broadest aromatic profile was achieved for the variants with sucrose and with galactose (Figure 2A). Both fermentation products gave strong honey-like and rose-like notes. On the other hand, when using fructose and glucose in a sour whey

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fermentation, the aroma was the weakest for all descriptors. In the case of the sweet whey culture (Figure 2B), again the sucrose variant had the most diverse odor profile, with strong honey-like, rose-like, and caramel-like notes. The use of glucose and fructose as components of the sour whey medium lowered the intensity of the rose-like and caramel-like notes, while the addition of galactose was the least appreciated by the panelists, who in this case ranked all the descriptors lowest. Taking these results into consideration, sucrose was selected as the carbon source that yielded the most pleasant aroma with strong honey and rose-like odor notes.

The presence of rose-like aroma is typical of certain types of cheeses and fermented food products, such as black tea and pumpernickel bread.[7,22−24] It has been well established that during L-phenylalanine transformation, honey-rose aroma compounds are produced, including phenylacetaldehyde, 2-phenylethanol, phenylacetic acid, and 2-phenylethylacetate. These compounds are formed in the Ehrlich pathway.[4,25,26] The resulting 2-phenylethanol can then be esterified to 2-phenylacetate.[25] An alternative pathway for reducing phenylacetaldehyde to 2-phenylethanol is oxidizing it to phenylacetic acid.[27] Although the formation pathway of these compounds is interdependent, they are rarely all present in one product at a time.[22,25] It should be noted that there are dependencies between the compounds arising in the Ehrlich pathway that affect the characteristics of the aroma. According to Whetstine et al.,[22] phenylacetaldehyde plays a more important role in shaping the rose aroma than does phenylacetic acid. However, it has been shown that phenylacetaldehyde, in combination with even a small amount of phenylacetic acid, is characterized by a much higher intensity of rose aroma than the sum of individual impressions of these compounds. This phenomenon is most pronounced at high concentrations of phenylacetaldehyde in the product.[22] According to Dunkel et al.,[17] phenylacetaldehyde and 2-phenylethanol are equally often recognized as key odorants as in 22.5 and 22.9%, respectively, of food products. Numerous studies of fermented products have shown that both those compounds are formed during fermentation and are present in the final product. For example, Schuh and Schieberle[23] identified the phenylacetaldehyde and 2-phenylethanol formed during fermentation of black tea leaves to have a concentration ratio of 0.3:1. On the other hand, in our research on ripened cheese, we found the concentration of phenylacetaldehyde and 2-phenylethanol with a ratio of 0.7:1, which correlated with the sensory analysis and honey-rose flavor.[7] Further analysis confirmed that it is *G. geotrichum* that is responsible for this biotransformation and for the formation of phenylacetaldehyde and 2-phenylethanol. This also reinforces the fact that phenylacetaldehyde has stronger aromatization power than 2-phenylethanol and therefore has higher potential to bring desirable rose-like aroma to fermented products.

In our study, the presence of L-phenylalanine in the medium as a component of whey and as a nutrient ingredient for *G. geotrichum* allows the formation of honey-like and rose-like compounds such as phenylacetaldehyde, 2-phenylethanol, phenylacetic acid, and 2-phenylethylacetate.

G. geotrichum grows over broad ranges of temperature and pH, although 25−30 °C and pH 5.0−5.5 are suggested as optimal.[28] We tested three different incubation temperatures (25, 30, and 35 °C) to find which is optimal for the growth of *G. geotrichum* and for the honey-rose aroma. The sensory profiles obtained were the broadest and had the strongest honey-like note for the cultures grown at 30 °C, for both sour and sweet whey variants. Panelists noted the presence of a strong rotten fruit note in the samples incubated at 25 °C, while at 35 °C, a very poorly perceptible aroma was obtained.

Comparing the effects of pH values of 3.0, 4.0, and 5.0 on the aroma showed that the most intense honey-rose odor profile was obtained by incubation on medium at pH 5.0, again for both types of whey. The aroma produced using the medium of pH 4.0 was about half as intense as at pH 5.0, while the culture at pH 3.0 had the least intense sensory profile.

The results for the individual descriptors are consistent with the assessments of general desirability for the variants we tested. In both sour and sweet whey cultures, samples with medium at pH 5.0 and those with an incubation temperature of 30 °C were most desirable by 6 to 7 points. In comparing the type of sugar in the medium, the highest values of desirability were achieved with sucrose in both whey variants. It should be noted that neither incubation temperature nor pH value caused as large a difference as did sugar type.

Based on the sensory evaluation and quantitative analysis of the aroma compounds in further experiments, the following were selected as the ideal culture conditions: sucrose in the medium, medium pH at 5.0, and an incubation temperature of 30 °C.

Sensory Evaluation of Bioreactor Cultures. Having determined the optimal culture parameters, the fermentation process was moved to a larger scale with a 2.3 L bioreactor using media with sour and sweet whey. In the first stage, the aroma compositions we obtained underwent extended sensory evaluation, the results of which are given in Figure 3; these

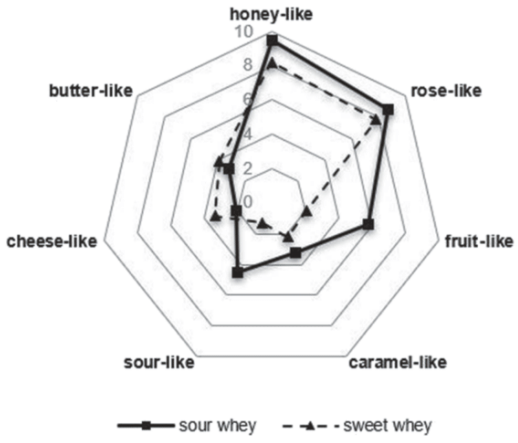

Figure 3. Sensory profile of post-fermentation products from bioreactor cultures with sour and sweet whey.

show that both compositions had strong honey and rose aromas, with a slight advantage for the sour whey. In addition, the sour whey culture had a more intense fruit-like aroma, while the sweet whey sample showed had a significantly less intense caramel-like note. Butter-like and cheese-like flavor notes were barely perceptible in either variant, whereas the sour whey culture showed a sour-like aroma of the medium intensity. The difference between the odor profiles of the bioreactor cultures and the flask cultures may be associated with the more carefully controlled conditions in the bioreactor, such as regarding aeration.

Identification of Key Aroma Compounds in a Post-Fermentation Product by Means of GC−O Analysis and

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Table 2. Key Odorants Identified in Post-Fermentation Products from Sour and Sweet Whey from the Bioreactor Culture

Compound[a]	Odor[b]	IR-DB 5[c]	IR-wax[c]	concentration (μg/kg)[d]		OT in water (μg/kg)[e]	OAV[f]	
				sour whey	sweet whey		sour whey	sweet whey
2,3-butanedione	buttery	670	991	550 ± 11	610 ± 12	15	37	41
acetic acid	vinegar	691	1445	250,120 ± 128	5126 ± 89	99,000	2.5	<1
3-methylbutanal	malty	695	948	45 ± 2	0.8 ± 0.05	0.5	90	1.6
3-methyl-1-butanol	fruity	719	1204	121,511 ± 498	1546 ± 57	980	131	1.6
butanoic acid	cheesy	836	1620	18,523 ± 212	17,450 ± 400	1000	18	17
3-(methylthio)-propanal	boiled potato	908	1458	51 ± 0.8	48 ± 0.7	0.43	119	112
dimethyl trisulfide	cabbage	985	1377	7 ± 0.05	6.8 ± 0.05	0.099	71	69
phenylacetaldehyde	honey	1080	1644	12,040 ± 131	7105 ± 95	4	3010	1776
2-phenylethanol	rosy	1124	1920	7090 ± 91	4125 ± 52	240	29	17
phenylacetic acid	honey	1260	2568	1725 ± 32	3218 ± 38	1000	1.7	3.2

[a]Compounds identified by comparison with reference compounds on the basis of the following criteria: retention index (RI), mass spectra obtained by MS (EI), and odor quality at the sniffing port. [b]Odor perceived at the sniffing port. [c]Retention indices on BD-5 and SUPELCOWAX 10 columns. [d]Mean of triplicates ± standard deviations. [e]OT: odor thresholds in water.[44] [f]OAV: odor activity values calculated by dividing the concentration of an analyte by its odor threshold value.

Calculation of OAVs. In the SAFE extracts prepared from samples obtained after bioreactor fermentation of sour and sweet whey mediums by *G. geotrichum*, 10 compounds were identified by GC−O analysis. All were then quantified, and the OAVs were calculated for them by dividing the concentration of the analyte by its odor threshold value. The results in Table 2 show that for both whey variants, the highest OAVs were recorded for phenylacetaldehyde, with a strong honey aroma (3010 for sour whey and 1776 for sweet whey, respectively), although this was not the compound with the highest concentration. This compound is characterized by a strong honey aroma and is formed by the Ehrlich pathway, which involves the transamination of L-phenylalanine to phenylpruvate, its decarboxylation to phenylacetaldehyde, and its reduction to 2-phenylethanol.[4,25,26] Its odor threshold in water is 60 times less than that of 2-phenylethanol; however, much more 2-phenylethanol with rose aroma is formed in the Ehrlich pathway. In our research, the 2-phenylethanol level was found to be 7090 μg/kg for samples with sour whey and 4125 μg/kg for sweet whey, which means that the concentration ratio of phenylacetaldehyde and 2-phenylethanol is 1.7:1 in both variants. As mentioned, this ratio ensures greater aromatization power, which is clearly seen in the OAVs that are over 100 higher for phenylacetaldehyde than for 2-phenylethanol. Additionally, our results show that phenylacetic acid is formed in the bioreactor; this is a third compound with a honey, rose-like aroma, which is derived in the Ehrlich pathway after oxidation of phenylacetaldehyde.[27] This means that phenylacetic acid is present in products that also contain phenylacetaldehyde and/or 2-phenylethanol or one of them as compounds formed in the same process, such as dairy products, fermented beverages, beans, and soy sauce.[7,29−32] We can observe here the ratio between the concentration of phenylacetaldehyde, 2-phenylethanol, and phenylacetic acid to be 1.7:1:0.2 in sour whey and 1.7:1:0.8 in sweet whey. In the fried cottage cheese from which *G. geotrichum* was isolated, these compounds were found in a ratio of 0.7:1:0.2 after 4 days of ripening, with the concentration of 2-phenylethanol at 1892 μg/kg, whereas in fermented cocoa beans, this ratio was 0.03:1:2.1 and 2-phenylethanol was at a concentration of 2100 μg/kg.[7,33] In the drink produced by fermentation of the wort by *Trametes versicolor*, all three compounds were found in a ratio of 5.2:1:2.7. The concentration of 2-phenylethanol in the analyzed product was 31 μg/L, and despite the highest OAV content in the aroma tested for phenylacetaldehyde, it was characterized by only a slight floral aroma.[29] It should be noted that phenylacetaldehyde, 2-phenylethanol, and phenylacetic acid are found in fermented products together but that their levels may vary greatly, depending on the type of the substrate and the conditions of the fermentation process.[27] An example is the traditional Chinese fermented red pepper paste that contains phenylacetaldehyde and 2-phenylethanol in a ratio of 0.05:1 (at a 2-phenylethanol concentration in the product of 129.22 μg/kg) and no phenylacetic acid at all.[34] However, it should be noted that in the case of both phenylacetaldehyde and 2-phenylethanol, the post-fermentation product with sour whey contained 1.7 times more of these compounds than in the case of sweet whey.

The aroma compounds discussed earlier are important odorants in the aroma produced by *G. geotrichum*; however, it should be noted that human perception of mixtures of various flavor compounds is not just the sum of their individual aroma notes.[17,35] It has been shown that mixtures containing more than four odorants are characterized by the loss of individual fragrance notes for each compound in order to create a specific perception of the entire aroma.[17] Most aromas of natural origin are complex mixtures of different odorants, as is the aroma produced by *G. geotrichum* on the test substrates.[35] Another example is rape honey in which 2,3-butanedione, with its butter aroma (typical of fermentation processes), is formed as a result of natural transformations; dimethyl trisulfide with its cabbage-like aroma and (*E*)-β-damascenone with its scent of cooked apples are also produced. These compounds are characterized by a relatively high OAV, but the aroma as a whole is not directly related to any of these descriptors.[36]

Another compound identified as one of the key odorants in post-fermentation products is 3-methyl-1-butanol, with its fruit aroma. This is the second most abundant compound (121,511 μg/kg) in samples based on sour whey and is characterized by a relatively high OAV (131), whereas in the variant with sweet whey, it is present at 1546 μg/kg, being the sixth most abundant compound. Its formation is associated with fermentation processes, and its concentration increases to a maximum of 5600 μg/kg during three-stage sourdough fermentation, which is part of the preparation of pumpernickel bread.[24] This compound is present in fermented beverages, such as wine and cider.[37−39] It is produced by the microorganisms associated with grapes grown for wine

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production. It is the substance found in the highest concentration in the aromas produced by *Paenibacillus* sp. and *Aureobasidium pullulans*, and the second highest in the aromas is produced by *Sporobolomyces roseus*.[37] In apple ciders, after fermentation by *Hanseniaspora osmophila* and its mixed culture with *Torulaspora quercuum*, 3-methyl-1-butanol was found in the concentration from 3.56 to 4.65 μg/L.[38] This compound is also produced by *Staphylococcus xylosus*, which gives its characteristic aroma to fermented meat products.[40] Other compounds identified in high concentrations in the cultures tested here are acetic acid and butanoic acid with their odors of vinegar and cheese, respectively. Acetic acid has been shown to be formed during enzymatic degradation in the fermentation process of fruit pulp.[33] Butanoic acid is also a characteristic fermentation product formed during the transformation of carbohydrates.[17] In addition, subjecting whey to controlled fermentation processes could be a way of producing acetic or butanoic acid.[12] In samples with sour whey, acetic acid is the key odorant found in the highest concentration, but due to its high OT value, this results in an OAV of 2.5—the lowest value after 2-phenylethanol. In the sweet whey variant, acetic acid is present at a concentration lower than the OT value, which gives OAV < 1. However, butanoic acid is present in a similar concentration in both sweet and sour whey cultures (at 18,523 and 17,450 μg/kg, respectively). Another compound identified as a key odorant is 2,3-butanedione, with its aroma of butter, which is present at 550 and 610 μg/kg in the sour and sweet whey variants, respectively. This compound is present in various types of cheese due to bacterial or mold fermentation, being a key aroma compound in fried cottage cheese, Lazur, Camembert, Cheddar, and Emmental cheeses.[7,9,22,41] The compound with the eighth highest concentration in both culture variants (51 and 48 μg/kg, respectively, for sour and sweet whey) was identified as 3-(methylthio)-propanal, which has a smell of boiled potatoes. This compound is characterized by a low OT value, which resulted in the third and second highest OAVs for the sour and sweet whey variants, respectively. Both 3-(methylthio)-propanal and 3-methylbutanal with its malty aroma (found at 45 and 0.8 μg/kg in the sour and sweet whey, respectively) are formed during Strecker degradation from methionine and leucine, respectively. This reaction can also be carried out by means of an enzymatic reaction in the Ehrlich pathway.[24,42] It has been demonstrated during the preparation of bread dough that increasing the amount of yeast and applying proper fermentation conditions (reduced temperature and shorter time) increased the content of 3-(methylthio)-propanal in the bread.[43] 3-Methylbutanal is present in high concentrations in soy sauce due to the formation during the fermentation process of large amounts of free amino acids, which are precursors of this compound.[32] The final key odorant determining the aroma produced by *G. geotrichum* molds is dimethyl trisulfide, with the fifth highest OAV for the sour whey and the third OAV for the sweet whey. The cabbage odor note of this compound was not strongly perceptible in the aroma studied, but it significantly affects the overall composition. According to the review on key odorants, the following compounds: acetic acid, butanoic acid, 2,3-butanedione, 3-(methylthio)-propanal, and 3-methylbutanal identified as key odorants in the post-fermentation product analyzed here contributed to the aroma of more than 25% of 227 food samples and therefore belong to the "generalist" group.[17]

Combined analysis of the results from GC−O analysis, from the OAVs, and from sensory evaluation show that phenylacetaldehyde is responsible for the honey note and 2-phenylethanol for the rose note in the aroma produced by the *G. geotrichum* mold. The occurrence of a delicate buttery note can be associated with 2,3-butanedione. 3-Methyl-1-butanol is a compound that is present in a higher concentration in cultures with sour whey, with this variant also characterized by a more intense fruity note, so the presence of this compound should be associated with the aroma. Acetic acid is responsible for the more intense sour aroma note in the bioreactor culture with sour whey, present in this case in a higher concentration than in the sweet whey. It is hard to link the rest of the odor descriptors with the specific aroma compounds responsible for their appearance, probably as a result of the combination of several compounds, individually described by other notes.

In summary, we determined the optimal conditions for culturing *G. geotrichum*, resulting in post-fermentation products with an intense honey-like, pleasant aroma. The screening of *G. geotrichum* strains and optimization of fermentation conditions were conducted with the aim of achieving the preferred ratio (>1) of phenylacetaldehyde to 2-phenylethanol in the Ehrlich pathway. Strain 32 was chosen in the case of both medium supplements, and the selected culture conditions were sucrose as the medium component, a pH of 5.0, and an incubation temperature of 30 °C. Further experiments in a 2.3 L bioreactor gave fermented products with strong honey-like, rose-like, and fruit-like aromas. The application of GC−O analysis and the calculation of OAVs allowed 10 key aroma compounds to be identified in the post-fermentation culture. Phenylacetaldehyde with its honey aroma had the highest OAV among all the key odorants in both the sour and sweet whey variants. In addition, in both culture variants, we determined the presence of phenylacetaldehyde and 2-phenylethanol, responsible for honey and rose odor notes, respectively, in a ratio of 1.7:1, which resulted in a much more intense aroma. However, the concentration of these compounds was 1.7 times higher in the product with sour whey than with sweet whey. In conclusion, our results show that fermentation with *G. geotrichum* on a medium with sour or sweet whey gives the possibility to obtain a post-fermentation product with strong honey-like and rose-like aromas due to the high ratio of phenylacetaldehyde to 2-phenylethanol formed by the Ehrlich pathway.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.0c03979.

TIC chromatogram of SAFE extract obtained from sour whey fermentation by *G. geotrichum* in a 2L bioreactor (PDF)

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■ ABBREVIATIONS

GC−O, gas chromatography−olfactometry; HS-SPME, headspace solid-phase microextraction; OAV, odor activity value; OT, odor threshold; GC−MS, gas chromatography−mass spectrometry; SIDA, stable isotope dilution assay

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Analysis of the Ability to Produce Pleasant Aromas on Sour Whey and Buttermilk By-Products by Mold *Galactomyces geotrichum*: Identification of Key Odorants

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Abstract: Currently, there is a growing demand for flavorings, especially of natural origin. It is worth paying attention to the biotechnological processes of flavor production, characterized by simplicity, high efficiency and relatively low cost. In this study, we analyzed the ability of the *Galac tomyces geotrichum* mold to transform by-products of the dairy industry: sour whey and buttermilk to complex flavour mixtures with pleasant, honey-rose aroma. Furthermore, the aroma complexity of the fermentation product has been carefully identified applying a sensomic approach involving the use of gas chromatography-olfactometry (GC-O), gas chromatography-mass spectrometry (GC-MS) and stable isotope dilution assay (SIDA) to identify and quantify aroma compounds. Based on the calculation of odor activity value (OAV), 13 key aroma compounds were present in both tested variants. The highest OAVs were found for phenylacetaldehyde (honey-like) in the buttermilk variant (912) and 2-phenylethanol (rose-like) in the sour whey variant (524). High values of this indicator were also recorded for phenylacetaldehyde (319) and 3-methyl-1-butanol with a fruity aroma (149) in the sour whey culture. The other compounds identified are 3-methylbutanal (malty), 2,3-butanedione (cheesy), isovaleric acid (cheesy), 3-(methylthio)-propanal (boiled potato), butanoic acid (vinegar), (*E*)-2-nonenal (fatty), ethyl furaneol (burnt sugar), dimethyl trisulfide (cabbage), and acetic acid (vinegar).

Keywords: whey; buttermilk; *Galactomyces geotrichum*; fermentation; aroma-active compounds; aroma biotechnology; SIDA; GC-O; OAV

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1. Introduction

Modern food production technologies shape the growing demand for flavorings. This is related to the shortening and simplification of processing, which may result in obtaining an aroma of the product with insufficient intensity. The aroma compounds can be obtained by chemical synthesis, extraction from natural sources, and biotechnological processes. Synthetic aroma compounds are characterized by high production efficiency, simplicity of the technology, and low price. However, they are less and less acceptable among consumers who prefer food additives of natural origin. Moreover, obtaining volatile compounds by means of chemical reactions is very often energy-consuming and harmful to the environment. The extraction of aromas from natural sources, on the other hand, is subject to several limitations, such as the impact of seasonality, climate changes, and political features on the availability of the raw material; the difficulty and time-consuming technologies, or a higher price. In the face of the growing demand for natural aromas, biotechnological processes are a promising alternative to traditional methods. These technologies include de novo synthesis and bioconversion of natural precursors using microbial cells or enzymes. Biotechnological production of flavors presents several important benefits when compared with the previously mentioned methods, such as high

production efficiency, simplicity of the technology, controllability, independence from many external factors, care for natural resources, relatively low production cost, and the possibility of managing industry by-products. However, the factors limiting these technologies are the possible variability of the composition of media containing ingredients of natural origin, the need to develop technologies for the recovery of aroma compounds in some cases, as well as the possibility of contamination of the culture by incomplete sterilization of the medium, contamination of the inoculum or careless handling during sampling and other necessary activities [1–4].

Food is a complex matrix for which several compounds are responsible for the aroma. Therefore, there is a growing interest in producing aroma mixtures rather than individual compounds, as they can more closely resemble the aroma of food resulting from natural transformations [5]. This approach also eliminates the need to implement time-consuming and costly processes to extract specific aroma compounds from microbial cultures. Potential applications of aroma compositions are food products whose aroma is not characterized by a sufficient intensity due to the shortening of fermentation processes, as in the brewing, dairy or baking industries. An important stage in developing new technologies for the biotechnological production of aroma mixtures is the determination of the key odorants in the resulting product due to the multitude of aroma compounds. Previous studies have shown that the best approach to fulfil this task is the application of sensomic concepts, which allows for careful identification of odor-active compounds responsible for the sensory properties and sensory acceptance of fermented products [5–7].

There has, in recent years, been a continuous increase in the consumption of milk and dairy products. The growing demand for these products generates an increase in their production, resulting in a greater supply of dairy industry by-products, including whey and buttermilk [8]. Whey is a yellowish liquid obtained during cheese production by separating the curd. Depending on the type of technology, there are two types of whey: sweet whey, formed after enzymatic coagulation, and sour whey, formed during the production of cottage cheese. Whey contains 93–94% of water, 4.5–6.0% of lactose, 0.6–1.1% of proteins, 0.8–1.0% of minerals, 0.05–0.9% of lactic acid, and 0.06–0.5% of fat, depending on the method of obtaining it [9,10]. Buttermilk is a cream-colored liquid, aqueous side stream obtained during butter production by separating the milk fat. This by-product contains 91–92% of water, 3.6–6.7% of lactose, 2.4–3.5% of proteins, 0.6–0.8% of minerals, 0.7% of lactic acid, and 0.5–1.5% of fat, depending on the method of obtaining it [11–13]. It should be noted that both whey and buttermilk contain L-phenylalanine, which is a precursor of rose-like aroma compounds formed in the Ehrlich pathway [14–16]. In this study, sour whey and buttermilk were analyzed, both in spray-dried form. This approach is due to the wide availability of these by-products in this form and the possibility of easier and longer storage and lower transport costs. The key factor, however, was the ability to sterilize the spray-dried medium components by exposure to UV radiation, which prevents changes in the final aroma due to thermal reactions. According to FAOSTAT [17], the global production of spray-dried sour whey and buttermilk in 2018 amounted to over 2.8 and almost 0.7 million tones, respectively. Over the ten years, an increase in production was observed in the amount of over 0.3 and 0.5 million tons, respectively.

Galactomyces geotrichum is a mold that is part of the natural microflora of raw milk and dairy products, affecting the chemical composition as well as nutritional and sensory properties of the resulting food products [18,19]. It should be noted that *G. geotrichum* mold is Generally Recognized as Safe (GRAS), so there is no risk of potential toxicity from this microorganism [20]. According to our recent studies, *G. geotrichum* is responsible for the formation of intense aroma with honey, rose and fruity odor notes on substrates with sweet whey and sour whey [21].

In the present studies, the ability of *G. geotrichum* mold to transform by-products of the dairy industry (sour whey and buttermilk) to complex flavour mixtures was investigated. This study is part of a larger experiment to analyze the aroma produced by *G. geotrichum* on different substrate variants containing dairy by-products [21]. The aroma composition

of obtained post-fermentation product was fully characterized using a sensomic approach including gas chromatography olfactometry (GC-O) analysis, solvent assisted flavour evaporation (SAFE) extraction, gas chromatography and mass spectrometry (GC/MS), and calculation of odor activity values (OAV).

2. Results and Discussion

2.1. Optimization of Culture Conditions

This experiment aimed to select conditions for the cultivation of *G. geotrichum* mold on media with sour whey and buttermilk, which would allow for the production of the pleasant honey-rose aroma of the highest possible intensity. Therefore, four variants of sugar added to the medium, three variants of the pH value, and three variants of the incubation temperature were analyzed. Each of the tested variants was subjected to a profile sensory analysis, including the evaluation of the intensity of four descriptors: honey-like, rose-like, fruit-like, and caramel-like, and general desirability, meaning whether the aroma is liked or not.

2.1.1. Type of Sugar

The influence of the addition of the following sugars on the sensory quality of the aroma produced by *G. geotrichum* was analyzed: sucrose, glucose, galactose, and fructose. Lactose was not included in this experiment because our preliminary studies showed that *G. geotrichum* mold is incapable of fermenting it. The results in Figure 1 illustrate that the variants with galactose as a component of the medium of both products and the variant with sucrose in the case of sour whey had the most extensive aroma profiles. The post-fermentation product with sour whey and sucrose as ingredients in the culture medium was subjected to a detailed analysis of key odorants in our previous studies [21]. The same variants were characterized by the highest general desirability (5.8). The fermentation product with sour whey and galactose gave strong honey-like (5.7), rose-like (3.8), and fruit-like (5.1) notes. In the case of the buttermilk product with the addition of galactose, the panelists did not find a strong fruit-like aroma but assessed the caramel-like note (4.6) as intense. The glucose variant had a similar odor profile but with a lower intensity. Moreover, the post-fermentation product with buttermilk and galactose was characterized by higher desirability (6.9) and intensity of the honey-like aroma note (7.4) than any sample with sour whey. The sensory profile of both fructose-containing variants was assessed as significantly less developed. The variant with sour whey and glucose and the variant with buttermilk and sucrose were characterized by the least intense aroma.

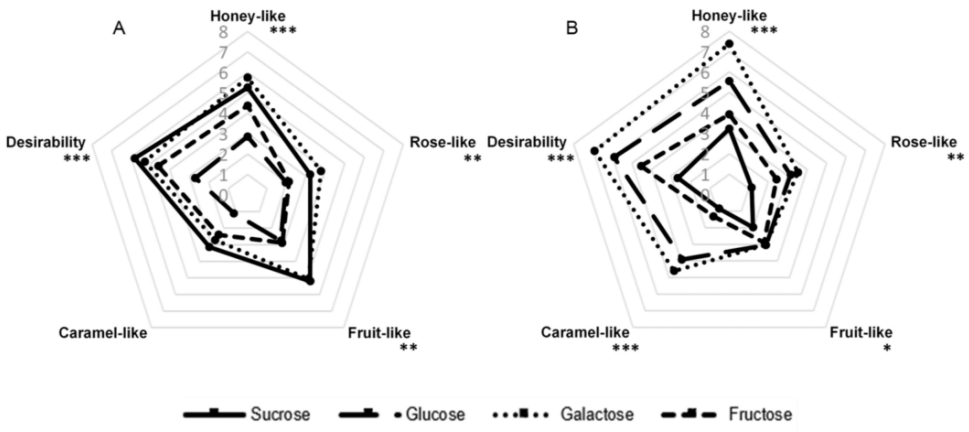

Figure 1. Sensory profiles of post-fermentation products from flask cultures with (**A**) sour whey and (**B**) buttermilk for various types of sugars. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

The number of CFUs was determined in all analyzed samples to investigate the influence of the amount of *G. geotrichum* biomass on the intensity of specific aroma notes. However, the results in Figure 2 show no correlation between the biomass concentration and the intensity of the aroma. In the case of sour whey, as a nutrient component, the samples with the most extensive and at the same very similar odor profile (galactose and sucrose) showed extremely different results in terms of CFU concentration of *G. geotrichum*. The variant with galactose was characterized by the lowest concentration of biomass (6.3 log CFU/mL), while the variant with sucrose—the highest (9.1 log CFU/mL). In the case of buttermilk, the variants with the broadest aroma profiles (galactose and glucose) were characterized by a high biomass concentration (8.2 and 9.2 log CFU/mL, respectively). However, a high number of CFU *G. geotrichum* was also found in the variant with sucrose (8.0 log CFU/mL), in which no high-intensity notes were found.

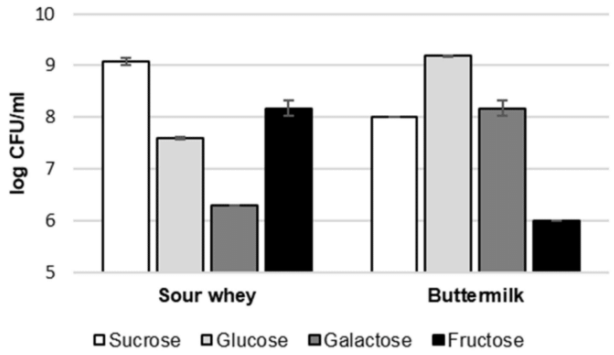

Figure 2. The concentration of biomass in post-fermentation products from flask cultures with sour whey and buttermilk for various types of sugars. Error bars represent ± SD from the replicates.

2.1.2. pH Value

G. geotrichum grows over a broad PH range; however, 5.0–5.5 are considered optimal [22]. We tested three variants of the PH of the media, determined at the beginning of the culture, to find the variant with the broadest aroma profile. The results in Figure 3 show that, for both sour whey and buttermilk cultures, the aroma of the highest intensity was observed at the PH value of 5.0. The aroma profile of the post-fermentation product formed on the sour whey medium with PH 4.0 was about half as intense, and the variant with PH 3.0 was characterized by the lowest intensity. In the case of buttermilk, panelists evaluated variants with PH 3.0 and 4.0 as poorly perceptible.

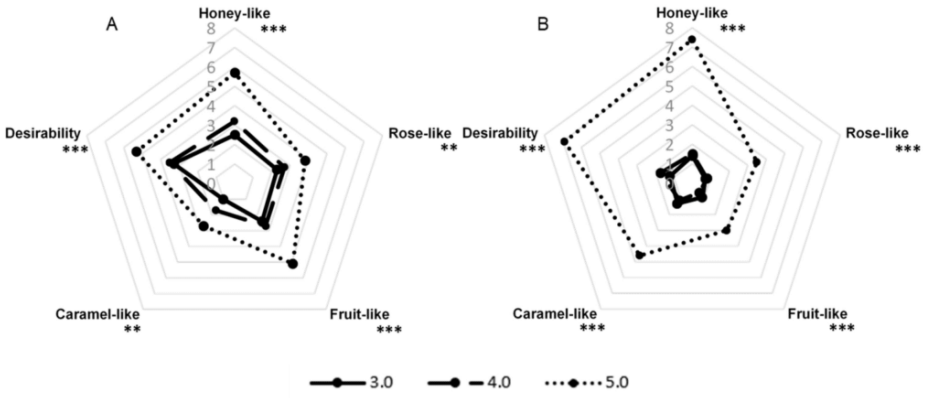

Figure 3. Sensory profiles of post-fermentation products from flask cultures with (**A**) sour whey and (**B**) buttermilk for various types of PH of the media. ** $p < 0.01$; *** $p < 0.001$.

2.1.3. Incubation Temperature

G. geotrichum has been shown to be able to grow over a wide temperature range of 5–38 °C during cheese production, with 25–30 °C being given as the optimal growth temperature [22]. The comparison of three variants of incubation temperatures (25, 30, and 35 °C) is presented in Figure 4. The results showed that the broadest aroma profile was obtained after a 7-day culture at 30 °C for sour whey and buttermilk product. After growing *G. geotrichum* on the sour whey medium at 25 °C, panelists noted an intense note of rotten fruit. The variants incubated at 35 °C in the case of sour whey and 25 °C in the case of buttermilk had a very slightly perceptible aroma.

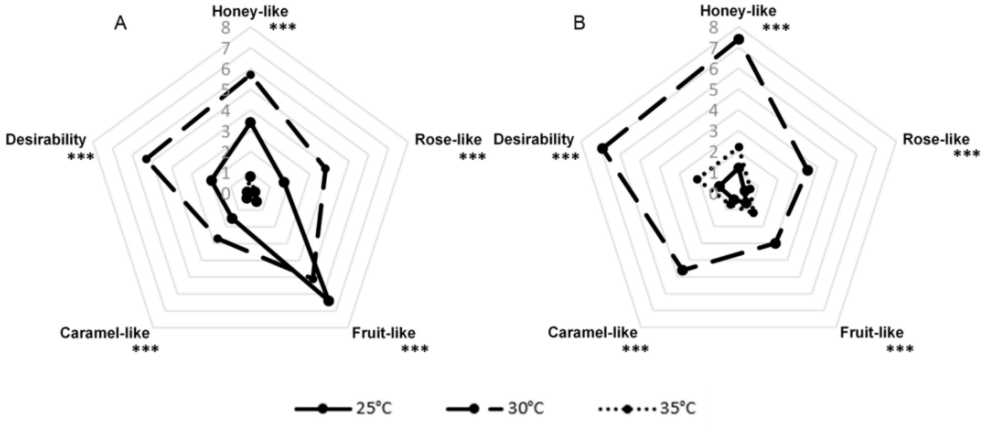

Figure 4. Sensory profiles of post-fermentation products from flask cultures with (**A**) sour whey and (**B**) buttermilk for various incubation temperatures. *** $p < 0.001$.

2.1.4. Determining of Culture Conditions

Based on the results of the sensory evaluation, the following optimal conditions were selected for the culture of *G. geotrichum* mold on substrates with sour whey and buttermilk: galactose as the type of sugar in the medium, medium PH at 5.0, and an incubation temperature of 30 °C.

2.2. Sensory Evaluation of Flask Cultures

After determining the optimal conditions for the cultivation of *G. geotrichum* on media with sour whey and buttermilk, flask cultures were carried out. The obtained post-fermentation products were subjected to a sensory evaluation, including additional odor descriptors, the results of which are presented in Figure 5.

The desirability of both products was highly appreciated by the panelists, despite the fact that their sensory profiles are very diverse. The slightly higher acceptability of the post-fermentation product with buttermilk (7.5) than the variant with sour whey (6.8) was probably due to the strong honey aroma desired by consumers. The variant with the addition of sour whey was characterized by a high intensity of honey-like (5.1), rose-like (4.0), fruit-like (5.1), butter-like (5.3), and cheese-like (4.5) notes. The panelists evaluated the intensity of the sour-like note (7.5) as the highest, while the caramel aroma (2.2) was the least perceptible. In the case of products with the addition of buttermilk, a much greater intensity of honey-like (7.6), caramel-like (5.2), and butter-like (8.3) notes were observed than in the case of sour whey variant. There was also a sour-like aroma (4.0) in this product. Rose-like (2.9), fruit-like (2.1), and cheese-like (2.0) notes were the least perceptible. The desirability of both products was highly appreciated by the panelists.

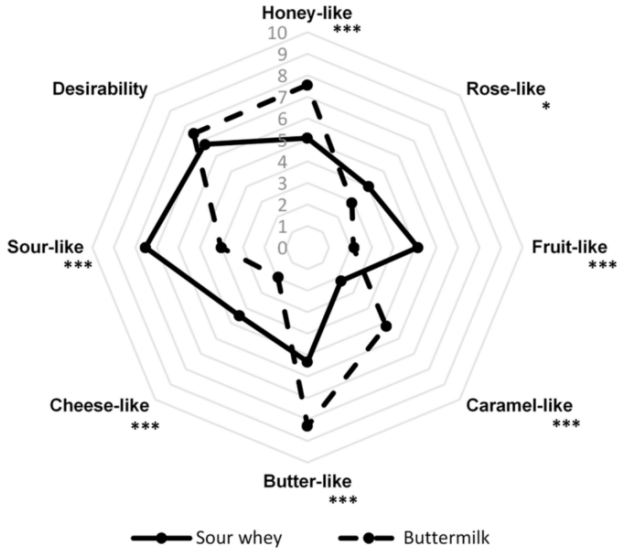

Figure 5. Sensory profiles of post-fermentation products from flack cultures with sour whey and buttermilk. * $p < 0.05$; *** $p < 0.001$.

The presence of a honey-rose aroma is found in many products, which includes fermentation processes, such as cheese, pumpernickel bread or traditional pastes [19,23–25]. It has been well established that the most frequently responsible for this aroma are compounds formed during the transformation of L-phenylalanine in the course of the Ehrlich pathway, such as phenylacetaldehyde, 2-phenylethanol, phenylacetate, and phenylacetic acid [15,26,27]. According to Dunkel et al. [5], phenylacetaldehyde and 2-phenylethanol have been identified as key odorants in over 50 different food products (51 and 52 respectively), such as alcoholic beverages, fermentation products or thermally treated foods. In previous studies, we have shown that 39 strains of *G. geotrichum* are capable of producing phenylacetaldehyde and 2-phenylethanol on a basic medium (without the addition of whey or buttermilk) [21]. The use of appropriate additives to the medium and the optimization of culture conditions allowed for obtaining the post-fermentation product with an extensive, pleasant honey-rose aroma profile.

2.3. Identification of Key Aroma Compounds in a Post-Fermentation Product Using GC−O Analysis and Calculation of OAVs

In order to fully characterize the post-fermentation products obtained in the course of biotechnological transformation, the key aroma compounds were identified using GC-O analysis and OAVs calculation. In SAFE extracts prepared from the samples obtained from the sour whey and buttermilk media after the flask cultures with *G. geotrichum* using the parameters defined during the culture optimization, 13 aroma compounds were identified during the GC-O analysis. Table 1 presents the identified compounds with the results of their quantitation and the OAVs. According to Schieberle [28], OAV can be used as an indicator of the aroma compound activity and is calculated by dividing the concentration of an analyte by its odor threshold (OT) value. Choosing an appropriate matrix for OT is crucial as it influences the release of volatile compounds. As the matrix of the post-fermented product contains mostly water, the OT used for calculation in our studies is also based on water. The calculation of OAVs allows for determining the influence of individual compounds on the overall aroma of a product [6,29–32]. The higher the value of this indicator, the greater the importance of the analyzed volatile in shaping the aroma. The results of the OAV calculations obtained in our studies indicate that all quantified compounds are characterized by OAVs greater than 1.0, which indicates their potential in the aroma formation of the analyzed products. It should be noted that 8 of the identified compounds (2,3-butanedione, acetic acid, 3-methylbutanal, butanoic acid, isovaleric acid,

3-(methylthio)-propanal, furaneol, and (*E*)-2-nonenal) belong to the group of compounds called "generalist", which are key odorants in more than 25% of food products [5]. In the sour whey samples, two compounds were found having OAVs ranging from 0 to 10, six compounds ranging from greater than 10 to 100, two ranging from greater than 100 to 500, and one compound ranging from greater than 500 to 1000. In the buttermilk variant, two compounds were found having OAVs ranging from 0 to 10, nine compounds ranging from greater than 10 to 100, and one compound ranging from greater than 500 to 1000. One compound was identified with a concentration below the detection limit in both variants, which made it impossible to calculate OAV.

Table 1. Key odorants identified in post-fermentation products from sour whey and buttermilk.

Compound [a]	Odor [b]	RI-DB 5 [c]	RI-Wax [c]	Concentration (µg/kg) [d]		OT in Water (µg/kg) [e]	OAV [f]	
				Sour Whey	Buttermilk		Sour Whey	Buttermilk
2,3-butanedione	buttery	670	991	370	680	15	25	45
acetic acid	vinegar	691	1445	127250	189780	99000	1.3	2.0
3-methylbutanal	malty	695	948	38	36	0.5	76	72
3-methyl-1-butanol	fruity	719	1204	146223	2730	980	149	2.8
butanoic acid	cheesy	836	1620	32040	13030	1000	32	13
isovaleric acid	cheesy	870	1665	19153	6286	490	39	13
3-(methylthio)-propanal	boiled potato	908	1458	13	11	0.43	30	26
dimethyl trisulfide	cabbage	985	1377	0.6	1.5	0.099	6	15
phenylacetaldehyde	honey	1080	1644	1276	3650	4	319	912
furaneol (4-hydroxy-2,5-dimethyl-3(2*H*)-furanone)	caramel	1090	2025	<LOD	<LOD	87		
2-phenylethanol	rosy	1124	1920	125650	4653	240	524	19
ethyl furaneol (2-ethyl-4-hydroxy-5-methylfurane-3(2H)-one)	burnt sugar	1135	2080	28	326	17	1.6	19
(*E*)-2-nonenal	fatty	1160	1527	3.4	3.5	0.19	18	18

[a] Compounds identified by comparison with reference compounds based on the following criteria: retention index (RI), mass spectra obtained by MS (EI), and odor quality at the sniffing port. [b] Odor perceived at the sniffing port. [c] Retention indices on DB-5 and SUPELCOWAX 10 columns. [d] Mean values based on three replicates with RSD value ≤11%. [e] OT: odor thresholds in water [33]. [f] OAV: odor activity values calculated by dividing the concentration of an analyte by its odor threshold value.

The results presented in Table 1 show that the compounds with the highest OAVs were 2-phenylethanol with rose aroma (524) and phenylacetaldehyde (319) in the sour whey variant, and phenylacetaldehyde with honey aroma (912) in the buttermilk post-fermentation product. It should be noted that 2-phenylethanol was found in the second highest concentration produced by *G. geotrichum* on the sour whey substrate. At the same time, phenylacetaldehyde did not have such a high concentration in any tested variants. This can be explained by 60 times higher phenylacetaldehyde odor threshold (in water) than 2-phenylethanol [33]. Phenylacetaldehyde can be formed either during heat treatment via *Strecker* reaction between dicarbonyl compounds and amino acids or via the *Ehrlich* pathway through transamination and decarboxylation of amino acid. In our studies, we have used the procedures limiting the possibility of Maillard reactions during the preparation of culture media and the fermentation process by *G. geotrichum* to ensure that the compounds in the analyzed post-fermentation product are formed strictly upon bioconversion. Microbial metabolism involves the formation of phenylacetaldehyde and 2-phenylethanol during the Ehrlich pathway involving transamination of L-phenylalanine to phenylpyru-

vate, followed by its decarboxylation to phenylacetaldehyde, which is then reduced to 2-phenylethanol [15,27]. Due to the differences in OT value between those two compounds, the higher the content of phenylacetaldehyde, at the expense of 2-phenylethanol, the higher aroma intensity of the final product. This has been exemplified by the post-fermentation product made from buttermilk. Although the concentration of 2-phenylethanol was higher than of phenylacetaldehyde (4653 and 3650 μg/kg, respectively), the OAV values showed that phenylacetaldehyde has the biggest influence on the aroma formation as its OAV was 912 while for 2-phenylethanol was only 19. These results could also explain the strongly perceived honey-like aroma in buttermilk post-fermentation product during sensory evaluation. In the case of the sour whey variant, the highest OAV value was obtained for 2-phenylethanol (524), which is most likely responsible for the rosy-like aroma perceived by the sensory panel. As for phenylacetaldehyde, based on obtained OAV (319), it is the second most intense compound in the sour whey post-fermentation product, responsible for the honey-like aroma.

The previously discussed compounds have the greatest influence on the aroma produced by the *G. geotrichum* molds due to the highest OAV values. However, the overall sensory characteristics of the resulting post-fermentation products also depend on the remaining 11 compounds identified. Another compound with a high OAV is 3-methyl-1-butanol with a fruity aroma. This compound was characterized by the highest concentration (146223 μg/kg) among all compounds identified in the aroma of the variant with sour whey. Based on its OT, 3-methyl-1-butanol reached OAV of 149 and most likely is responsible for the strong, fruity aroma in sour whey post-fermentation product. In the buttermilk based product, 3-methyl-1-butanol was present at a lower concentration of 2730 μg/kg, resulting in an OAV of 2.8 and a much less intensive fruity aroma in the sensory profiles. 3-methyl-1-butanol is produced during the fermentation process in beverages such as wine and cider [34–36]. Moreover, in the case of wine, its formation is related to the microflora naturally present on the grapes [35]. 3-methyl-1-butanol is also produced during the fermentation of sourdough, e.g., in a three-stage process during the production of pumpernickel bread [24]. The next aroma active compound, 3-methylbutanal, with a malty odor was found in both fermentation products at similar concentration levels: 38 μg/kg for sour whey and 36 μg/kg for the buttermilk variant. This compound is known to have a relatively low OT value (0.5 μg/kg in water). Therefore, it has been identified as a key odorants in many food products, such soy sauce, beer, and bakery products such as rye bread and pumpernickel bread [5,24,32,37]. Both 3-methyl-1-butanol and 3-methylbutanal are formed during the transformation of leucine [5,32,38]. 2,3-butanedione is the compound responsible for the strong buttery aroma in samples with buttermilk, where it was present at almost twice the concentration (680 μg/kg) than in the post-fermentation product with sour whey (370 μg/kg). This compound is formed during carbohydrate metabolism of a typical microflora of dairy products fermented by bacteria or molds, such as fried cottage cheese, Camembert, Cheddar, Emmental, and Lazur [5,19,25,39,40]. Two compounds with cheesy odor notes were found in the analyzed products: isovaleric acid and butanoic acid. In the variant with sour whey, which showed a stronger cheesy aroma, they were present at higher concentrations (19153 μg/kg and 32,040 μg/kg, respectively) than in the case of buttermilk (6286 and 13,030 μg/kg). This resulted in the corresponding OAV's; 39 and 32 for sour whey post-fermentation product and 13 and 13 for buttermilk one. These compounds can be formed by microbial metabolism—isovaleric acid is formed during the transformation of amino acids (isoleucine and leucine) and butanoic acid during the transformation of carbohydrates and triglycerides [5,41]. When considering the methods of whey management, Prazeres et al. [10] presented the use of controlled whey fermentation processes to produce butanoic and acetic acid. Acetic acid was also found in both tested variants. This compound was present in the post-fermentation product with sour whey and buttermilk, in high concentrations (127,250 and 189,780 μg/kg, respectively); however, due to the very high value of OT, it had the lowest impact on the overall odor profiles. The seventh compound with the highest OAV in the sour whey culture and the

fourth in the buttermilk variant was 3-(methylthio)-propanal with a boiled potato aroma. This volatile is formed during the transformation of methionine, also as part of microbial metabolism [5]. Another key odorant in order of OAVs is (*E*)-2-nonenal with a fatty smell, found in both the sour whey and buttermilk variants at 3.4 and 3.5 µg/kg, respectively. This compound has been already identified in a drink prepared from Darjeeling black tea or wheat bread, and is formed from unsaturated fatty acids by oxidation of fats. Two compounds are responsible for the caramel flavor in the buttermilk variant: ethyl furaneol (4-hydroxy-5-ethyl-2-methyl-3(2H)-furanone) with the aroma of burnt sugar and furaneol (4-hydroxy-2,5-dimethyl-3(2H)-furanone) with the aroma of caramel. Ethyl furaneol was found in this product at a concentration of 326 µg/kg with OAV 19, while the sour whey variant contains 12 times less of this compound. This volatile is one of the 12 most important odorants that shape the flavor of Japanese soy sauce, and biosynthesis by yeast was indicated as its method of production, suggesting the pentose-phosphate cycle involved in this process [32,42]. This compound is also found in meat sauce, which belongs to the same group of foods as soy sauce [43]. Furaneol was identified in the analyzed samples at the GC-O analysis stage, but the concentrations of this compound turned out to be below the limits of detection. Based on the Dunkel et al. review [5], this compound is a key odorant in 40.5% of foods, contributing to the aroma of soy sauce, various types of bread, and cocoa beans, among others [24,30,32,37]. Furaneol is produced by Maillard reactions and is found in many products subjected to thermal processes; however, Hecquet et al. [44] showed that it can also be biosynthesized by microorganisms [45]. The last identified compound is dimethyl trisulfide with a cabbage odor, which was found in the analyzed samples at the lowest concentrations of the 13 key odorants, 0.6 µg/kg for the sour whey variant and 1.5 µg/kg for buttermilk and OAVs od 6 and 15, respectively. However, no such odor notes have been identified in the post-fermentation products obtained.

The aroma profiles of the analyzed post-fermentation products can be combined with the presence of specific compounds. However, it should be noted that the overall aroma of a mixture of many compounds is not just the sum of the intensity of their individual aroma descriptors [5,46]. The sense of aroma is influenced by olfactory coding (still poorly understood) and olfactory perception, the understanding of which has been greatly improved in recent years [47]. It has been shown that, for as little as four compounds that create the aroma mixture, a loss of their individual aroma notes can be observed. In the analyzed post-fermentation products with sour whey and buttermilk, 13 key odorants were identified. Therefore, they can be called a complex mixtures of aroma compounds, similarly to most aromas of natural origin, which is why the issue of their perception is important [5]. For example, phenylacetaldehyde itself has a honey-like aroma, while when found in cheese in the presence of phenylacetic acid, it contributed to the formation of rose-like/floral notes of greater intensity [25]. Therefore, in addition to analyzing the combination of specific aroma notes with specific compounds, it is so important to perceive the aroma mixture as a whole.

3. Materials and Methods

3.1. Chemicals

Yeast extract, sucrose, glucose, and medium with chloramphenicol were obtained from BTL (Łódź, Poland). Galactose was purchased from Acros Organic (Geel, Belgium). Citric acid, $Na_2HPO_4 \cdot 2H_2O$, and $MgCl_2$ were obtained from POCH (Gliwice, Poland). Fructose was obtained from Chempur (Piekary Śląskie, Poland). L-Phenylalanine, lactic acid, sodium sulfate, ethyl acetate, dichloromethane, and diethyl ether were purchased from Sigma-Aldrich (Poznań, Poland). Inulin was obtained from Hortimex Plus (Konin, Poland). Spray-dried sour whey and buttermilk were purchased from Laktopol (Suwałki, Poland). The following reference aroma compounds were purchased from Sigma-Aldrich (Poznań, Poland): 2,3-butanedione, acetic acid, 3-methylbutanal, 3-methyl-1-butanol, butanoic acid, isovaleric acid, 3-(methylthio)-propanal, dimethyl trisulfide, phenylacetaldehyde, furaneol, maltol, 2-phenylethanol, ethyl furaneol, (*E*)-2-nonenal, and phenylacetic acid.

The following stable isotopes were obtained from AromaLAB (Freising, Germany): [$^{13}C_4$] 2,3-butanedione, [$^{13}C_1$] acetic acid, [$^{2}H_3$] 2-methylbutanal, [$^{2}H_2$] 3-methyl-1-butanol, [$^{2}H_7$] butanoic acid, [$^{2}H_2$] isovaleric acid, [$^{2}H_5$] 3-(methylthio)-propanal, [$^{2}H_6$] dimethyl trisulfide, [$^{2}H_5$] phenylacetaldehyde, [$^{13}C_2$] furaneol, [$^{2}H_3$] maltol, [$^{2}H_5$] 2-phenylethanol, [$^{2}H_2$] (*E*)-2-nonenal, and [$^{2}H_8$] naphthalene.

3.2. Microorganisms

The strain 32 *G. geotrichum* was isolated from Wielkopolski fried cottage cheese produced in the vicinity of Poznań in Poland during the ripening stage. Identification of the isolated strain was performed by amplification of the 18SrRna coding sequence. The strain was protected by lyophilization to standardize the inoculum and then deposited in the microbial culture collection of the Food Volatilomics and Sensomic Group.

Preparation of the *G. geotrichum* Strain

Strain 32 was lyophilized to standardize inoculum concentration. Shake flask cultivations were carried out in four 300 mL Erlenmeyer flasks with 100 mL of base medium containing per liter (modified, based on Grygier et al. [48]) 22.8 g of $Na_2HPO_4 \cdot 2H_2O$, 10.3 g of citric acid, 0.5 g of $MgCl_2$, and 0.17 g of yeast extract. The medium was sterilized, and then sucrose and L-phenylalanine were added to it at a concentration of 60 g/L and 21 g/L, respectively. These ingredients were previously exposed to UV radiation for 30 min. This approach aimed to prevent changes in the aroma profile of the products due to the influence of high temperature on the components of the medium. The medium used was also employed in all subsequent experiments. Each flask was inoculated with 10 µL of *G. geotrichum* strain 32, which had previously been revived by inoculating the agar slant with chloramphenicol (incubation at 30 °C for 72 h under aerobic conditions). Fermentation in flasks with analyzed *G. geotrichum* strain was carried out in a GFL 1086 water bath (GFL, Lauda-Königshofen, Germany) at 30 °C for 7 days with shaking (150 rpm). After the fermentation process was completed, the cultures were centrifuged (Biofuge Primo R, Heraeus, Hanau, Germany) for 10 min at 2012.4× *g* (3000 rpm) and 20 °C. The *G. geotrichum* precipitate was resuspended in 400 mL of the mixture of base medium and 10% inulin solution (1:1, *v*/*v*). To determine the number of colony-forming units (CFUs), the culture was sequentially diluted and plated onto agar plates with chloramphenicol, which were incubated for 72 h in 30 °C. After incubation of the agar plates, the CFUs were counted, and the number of colonies per milliliter of culture was calculated. The experiment was performed in triplicate. The freeze-drying process was conducted in a Beta 1–16 freeze dryer (Martin Christ, Osterode am Harz, Germany). The process was initiated with a freezing step at −35 °C for 2 h, followed by the main drying stage at 15 °C for 20 h, and the final drying at 22 °C (5 h). *G. geotrichum* lyophilisates were collected in glass jars under a nitrogen atmosphere.

3.3. Determining the Optimal Culture Conditions

The culture conditions were optimized by analyzing the influence of the type of sugar, the PH of the medium and the incubation temperature on the sensory profile of the two variants of the aroma produced by *G. geotrichum*. Both variants were prepared on the base medium. The first one was enriched with sour whey and the second one with buttermilk (spray-dried, at a concentration of 130 g/L). Moreover, at this stage, citric acid was replaced as a component of the nutrient medium with lactic acid, which occurs naturally in both acid whey and buttermilk [9,11]. This approach was aimed at mapping the natural environment of the development of *G. geotrichum*—dairy products [18,19]. Lactic acid was used to adjust the PH of the culture to a predetermined value in the final step of the medium preparation. This action was performed under sterile conditions in the presence of litmus papers as PH indicators. Flask cultures were carried out in 300 mL Erlenmeyer flasks with 200 mL of different medium variants, in triplicate. Each flask was inoculated with 3.4×10^6 CFU of lyophilized *G. geotrichum* strain.

3.3.1. Type of Sugar

The influence of four types of sugar (sucrose, glucose, galactose, and fructose) on the aroma profile of the post-fermentation products was analyzed. In order to avoid the influence of high sterilization temperatures on aroma profiles of the resulting products, each type of sugar was exposed to UV radiation for 30 min and then added to the sterile medium at a concentration of 60 g/L. The PH of the medium was adjusted to 5.0 prior to inoculation with *G. geotrichum*. Flask cultivations were carried out in a water bath at 30 °C for 7 days with shaking (150 rpm). After fermentation, the number of colony forming units (CFU) in each analyzed variant was determined. The sample was successively diluted and plated on chloramphenicol agar plates in triplicate. The plates were incubated for 72 h at 30 °C, and then the CFU were counted to calculate the number of *G. geotrichum* colonies per milliliter of sample.

3.3.2. pH Value

The effect of the PH of the medium on the aroma produced by *G. geotrichum* was examined by fermentation on three variants of media: PH 3.0, 4.0, and 5.0. The type of sugar in the medium was the one selected when optimizing the culture conditions in this direction. The fermentation process was carried out in a water bath at 30 °C for 7 days with shaking (150 rpm).

3.3.3. Incubation Temperature

The influence of the incubation temperature on the aroma profile of the post-fermentation products was analyzed by cultivating the *G. geotrichum* mold in three temperature variants: 25, 30, and 35 °C. The type of sugar in the medium was the one selected when optimizing the culture conditions in this direction, and the pH value was 5.0. Fermentation was carried out in a water bath for 7 days with shaking (150 rpm).

3.4. Sensory Evaluation

Descriptive sensory evaluation was carried out by ten experienced panelists experienced in conducting this type of research. The odor descriptors assessed were selected from the basic flavor descriptive language (Givaudan Roure Flavor [49]) and were determined in preliminary tests. The sensory analysis included the following odor descriptors: honey-like, caramel-like, rose-like, and fruit-like. The general desirability of the aroma produced by *G. geotrichum* was also analyzed. Sensory analysis was performed by scoring odor descriptors on a 10 cm linear scale, with the beginning labeled "none" and the end labeled " very strong". Panelists were presented with 20 g of samples obtained after fermentation carried out in all analyzed variants of culture conditions. The samples were placed in 100 mL glass containers and maintained at room temperature during analysis. The sensory evaluation of samples obtained from flask cultures after fermentation under the conditions obtained in the optimization process was carried out in the same way, except that, in addition to the previously mentioned aroma notes, the following descriptors were also analyzed: sour-like, butter-like, and cheese-like. The results were converted into numerical values for data analysis. The evaluations were performed in triplicate in separate profile sessions. The values given by panelist were averaged and analyzed by *t*-test. Significant differences were discussed in the text.

3.5. Flask Cultures

In order to obtain post-fermentation products on sour whey and buttermilk substrates by *G. geotrichum* mold, flask cultures were carried out using culture parameters determined in relation to the results obtained during the optimization of the culture conditions. The remaining aspects concerning the preparation of the culture medium and the course of fermentation were analogous to the optimization of the culture conditions. Shake flask cultivations were performed in triplicate for each analyzed variant.

3.6. Extraction of Volatile Compounds from Post-Fermentation Product Using the Solvent-Assisted Flavor Evaporation (SAFE) Method

The post-fermentation products obtained by culturing the *G. geotrichum* mold on sour whey and buttermilk media were subjected to extraction to isolate the odor-active compounds. The SAFE method described by Engel et al. [50] was used for this. Samples (50 g) were mixed with dichloromethane (100 mL) and spiked with the internal standard [$^2H_8$] naphthalene (25 µg). The samples prepared in this way were shaken for 2 h on a horizontal shaker to extract volatiles, which were then isolated using the SAFE method. The next step was to dry the obtained extracts under anhydrous sodium sulfate and finally concentrate them to approximately 500 µL using a Kuderna Danish concentrator (Sigma-Aldrich, Poznań, Poland).

3.7. GC-O and GC-MS Analysis

3.7.1. Gas Chromatography-Olfactometry (GC-O)

Extracts obtained using the SAFE method underwent GC−O analysis to identify odor-active compounds, using an HP 5890 chromatograph (Hewlett-Packard, Wilmington, DE, U.S.A.) with two columns of different polarities: SPB-5 (30 m × 0.53 mm × 1.5 µm) and SUPELCOWAX 10 (30 m × 0.53 mm × 1 µm) (Supelco, Bellefonte, PA, U.S.A.). In order to select compounds with perceptible odor notes, three panelists sniffed GC-effluent, thus indicating aroma-active regions in the analyzed samples. The effluent was divided between the olfactometry port with humidified air as a makeup gas and a flame ionization port using a Y splitter in GC. The operating conditions for the SPB-5 were as follows: an initial oven temperature of 40 °C (1 min) raised at 6 °C/min to 180 °C and at 20 °C/min to 280 °C. For the SUPELCOWAX 10 column, the operating conditions were as follows: an initial oven temperature of 40 °C (2 min) raised to 240 °C at a 6 °C/min rate and held for 2 min isothermally. SAFE extracts (2 µL) were injected into the GC column using the splitless mode. For all peaks and aroma notes with specific retention times, retention indices were calculated to compare results with those obtained by GC−MS and literature data. For each compound, the retention indices (RIs) were calculated using a homologous series of C7−C24 n-alkanes.

3.7.2. Gas Chromatography-Mass Spectrometry (GC-MS)

After GC-O analysis, odor-active compounds were identified and quantified using a 7890A GC (Agilent Technologies, Santa Clara, CA, USA.) coupled to a 5975C MSD (Agilent Technologies, Santa Clara, CA, USA.). The apparatus was equipped with two columns: SUPELCOWAX 10 (30 m × 0.25 mm × 0.25 µm) and an SLB-5ms (30 m × 0.25 mm × 0.5 µm) (Supelco, Bellefonte, PA, USA). The following operating conditions were used: helium flow of 32.2 cm/s and temperature programs the same as for the GC-O. Mass spectra were recorded in an electron impact mode (70 eV) in a scan range of m/z 33−350. The analyzed volatiles were identified by comparing their mass spectra, RIs, and odor notes on two columns of different polarities with respective standard compounds, National Institute of Standards and Technology (NIST) 09 Mass Spectral Library and literature data.

3.8. Quantitation by Stable Isotope Dilution Assays (SIDA)

The SIDA method was used during the quantitation of all identified aroma compounds. Dilutions of stock internal standards of the labeled isotopes for each identified compound (prepared in diethyl ether) were added to samples at similar concentrations to the relevant analyte present in the post-fermentation products. Only in the case of 3-methylbutanal was its direct isotopologue not available; therefore, 2-methylbutanal was used, which in our opinion, is the compound showing the greatest similarity. Odor-active compounds were then extracted and isolated from the samples by the SAFE method, and the obtained extracts were analyzed by GC-MS. During the analysis, the intensity of the individual ions was monitored and is shown in Table 2 for each of the identified aroma compounds. Response factors were calculated in the standard mixture of labeled and unlabeled compounds at a

known concentration of 500 ppb for all analyzed volatiles. The response factors were then used to apply corrections. Using the analyte peak areas and the corresponding internal standard, the concentrations for all analyzed aroma compounds were calculated, from which the concentrations obtained for the blank samples were then subtracted.

Table 2. Labeled standards and quantitation ions used for SIDA (stable isotope dilution assay) concentration calculations of 13 key odorants present in the post-fermentation products.

Compound [a]	Quant. Ions [b] (*m*/*z*) [b]	Labeled Standards [c]	Ion IS (*m*/*z*) [d]
2,3-butanedione	86	[$^{13}C_4$] 2,3-butanedione	90
acetic acid	60	[$^{2}H_3$] acetic acid	63
3-methylbutanal	86	[$^{2}H_3$] 2-methylbutanal	89
3-methyl-1-butanol	70	[$^{2}H_2$] 3-methyl-1-butanol	72
butanoic acid	73	[$^{2}H_7$] butanoic acid	77
3-methyl butanoic acid	87	[$^{2}H_2$] 2-methyl butanoic acid	89
3-(methylthio)-propanal	104	[$^{2}H_5$] 3-(methylthio)-propanal	107
dimethyl trisulfide	126	[$^{2}H_6$] dimethyl trisulfide	132
phenylacetaldehyde	120	[$^{2}H_5$] phenylacetaldehyde	125
furaneol (4-hydroxy-2,5-dimethyl-3(2*H*)-furanone)	128	[$^{13}C_2$] 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone	130
2-phenylethanol	122	[$^{2}H_5$] 2-phenylethanol	127
ethylfuraneol (2-ethyl-4-hydroxy-5-methylfurane-3(2H)-one)	125	[$^{13}C_2$] 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone	130
(E)-2-nonenal	140	[$^{2}H_2$] (E)-2-nonenal	142

[a] Quantified compounds; [b] Ions used for quantitation of analytes; [c] orresponding labeled standards used for quantitation; [d] Ions of internal standards (labeled isotopes) used for quantitation.

Finally, the odor activity values (OAVs) were calculated by dividing the concentration of a given analyte by the value of its odor threshold (OT) determined in water.

4. Conclusions

In conclusion, determining the optimal conditions for the culture (galactose as the type of sugar in the medium, medium PH at 5.0, and an incubation temperature of 30 °C) allowed for obtaining a high-intensity honey-rose aroma mixture with a pleasant aroma and high desirability, assessed by sensory panelists. While determining the optimal culture conditions, an analysis of the biomass concentration of variants containing different types of sugar in the medium was carried out. The results of this experiment showed that there is no correlation between the concentration of *G. geotrichum* biomass and the intensity of the aroma produced, which allows us to conclude that the sensory profile of the aroma produced is dependent on the culture conditions.

Using GC-O and calculating OAVs, 13 key odorants were identified in products obtained after fermentation of sour whey and buttermilk media by *G. geotrichum* mold. The sour whey post-fermentation product was also characterized by intense fruit-like and sour-like aroma notes. The compounds with the highest OAVs in this variant were the following compounds: 2-phenylethanol (rosy), phenylacetaldehyde (honey), 3-methyl-1-butanol (fruity) and 3-methylbutanal (malty), and isovaleric acid (cheesy). In the variant with buttermilk, the presence of intense caramel-like and buttery notes was found. In this product, the compounds with the highest OAVs were as follows: phenylacetaldehyde (honey), 3-methylbutanal (malty), and 2,3-butanedione (buttery). The presence of the honey-rose aroma of both culture variants is determined by the phenylacetaldehyde and 2-phenylethanol content in the sour whey product and the phenylacetaldehyde in the case of buttermilk.

The obtained results show that the fermentation process of the substrates with sour whey and buttermilk by *G. geotrichum* under certain conditions allows to obtain an aroma

mixture with an intense, pleasant aroma of natural origin. This indicates the possibility of using *G. geotrichum* mold to enrich the aroma of foods in many branches of the fermentation industry.

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III

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The Combined Effect of Lactic Acid Bacteria and *Galactomyces geotrichum* Fermentation on the Aroma Composition of Sour Whey

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Abstract: The increase in demand for food flavorings due to the shortening and simplification of food production technology also entails an increase in the demand for new technologies for their production. The biotechnological production of aromas is a solution characterized by a high efficiency, an independence from environmental factors and a relatively low cost. In this study, the influence of the implementation of lactic acid bacteria pre-fermentation into the production of aroma compounds by *Galactomyces geotrichum* on a sour whey medium on the intensity of the obtained aroma composition was analyzed. The monitoring of the culture in terms of biomass buildup, the concentration of selected compounds, and the pH resulted in the confirmation of interactions between the analyzed microorganisms. The post-fermentation product underwent a comprehensive sensomic analysis for the identification and quantification of the aroma-active compounds. The use of gas chromatography−olfactometry (GC−O) analysis and the calculation of odor activity values (OAVs) allowed 12 key odorants to be identified in the post-fermentation product. The highest OAV was found for phenylacetaldehyde with a honey odor (1815). The following compounds with the highest OAVs were 2,3-butanedione with a buttery aroma (233), phenylacetic acid with a honey aroma (197), 2,3-butanediol with a buttery aroma (103), 2-phenylethanol with a rosy aroma (39), ethyl octanoate with a fruity aroma (15), and ethyl hexanoate with a fruity aroma (14).

Keywords: sour whey; *Galactomyces geotrichum*; LAB; fermentation; aroma-active compounds; aroma biotechnology; stable isotope dilution assay; GC-O; OAV

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1. Introduction

Lifestyle changes, combined with the developments in food production technology nutrition in recent years, shape changes in the eating habits of people around the world. Consequently, there is a growing demand for ready-to-eat and ready-to-cook food, as well as ready-to-process food, which shortens the process of preparing meals at home. Another noticeable tendency in the food production market is the simplification and shortening of technological processes. The effect of the changes described above is an increase in the demand for food flavorings, which are necessary to provide a sufficiently intense aroma in processed foods. According to IMARC [1], the global food flavors market reached USD 15.6 billion in 2021, with an estimated value of USD 5 billion higher in 2027.

With the growing demand for food flavorings, there is a need to develop new technologies for their production. Particular emphasis is placed on the development of new technologies for the production of natural-origin flavors, which are currently the most acceptable additives for improving the aroma of food in connection with the growing nutritional awareness of consumers. The growing nutritional awareness of consumers is the result of promoting the healthcare trend. Consequently, food is no longer perceived

only in terms of providing basic nutritional properties but also in terms of its impact on vitality. Consumers prefer natural food additives over synthetic ones due to their perception as healthier, the possibility of giving food additional functional properties, and a more sustainable and ecological production process [2–4]. The traditional extraction of aroma compounds from natural sources (e.g., plants) may not be sufficient to meet the growing demand for food flavors due to suffering from difficulties, such as seasonality, climate-dependent availability, complexity, time-consuming production technologies, and the resulting relatively high price. Therefore, special attention should be paid to the biotechnological processes of obtaining aroma compounds of a natural origin. These processes include de novo synthesis and the bioconversion of natural-origin precursors by microorganisms or enzymes. Biotechnological processes for the production of aroma compounds are characterized by a higher efficiency, the simplicity of the technology, a high degree of controllability, a care for natural resources, and relatively low production costs, as well as an independence from environmental and sociopolitical factors. Biotechnological processes also offer the possibility of using industrial by-products, making such processes ecological. The main factors limiting the biotechnological production of aroma compounds are related to the possible contamination of the culture. This can be caused by an incomplete sterilization of the medium, a careless implementation of key culture steps or sample handling, and medium composition variability. In addition, in the case of this type of process, it may be necessary to develop technology for the recovery of aroma compounds [2,3,5,6]. However, there is currently a growing interest in aroma mixtures rather than single-aroma compounds in the food industry, which in many cases eliminates the need to develop such technologies. This approach allows for a better reproduction of the natural food aroma, which is usually very complex for any food product [7].

Galactomyces geotrichum mold is a natural microflora of dairy products, which affects their nutritional and sensory values [8,9]. *G. geotrichum* is a species referred to as a yeast-like fungus—in the literature it can also be found as *Geotrichum candidum*, an anamorphic form of the old *Galactomyces geotrichum/Geotrichum candidum* complex [10,11]. In our previous studies, we have shown that the strain of *G. geotrichum* isolated from fried cottage cheese has the ability to produce an intense honey–rose–fruit aroma during 7-day cultures on media with sweet whey, sour whey, or buttermilk and a L-phenylalanine supplement [12,13]. The aroma profile of the obtained products resulted mainly in an unusual ratio of phenylacetaldehyde and 2-phenylethanol, which, depending on the culture conditions, is close to or greater than 1: 1. The influence of this phenomenon on the aroma is due to the lower value of the phenylacetaldehyde odor threshold (OT) compared to 2-phenylethanol (60 times lower in water, 10 times lower in oil, and 4 times lower in starch) [14,15]. Moreover, *G. geotrichum* mold is Generally Recognized as Safe (GRAS); therefore, it can be considered in the context of searching for new technologies for enriching food aromas [16].

Lactic acid bacteria (LAB) are part of the microflora of the environment in which the *G. geotrichum* mold naturally occurs. In the fried cheese from which the strain of *G. geotrichum* analyzed in this study was isolated, LAB are responsible for the acidification of milk in the production process [8]. During lactic fermentation, LAB ferment lactose, providing an additional source of carbon for the analyzed molds that are unable to utilize this disaccharide. Moreover, low-molecular-weight metabolites of LAB may be present in the sour whey, which is a component of the culture medium used in this study. It is related to the process of producing sour whey, which is a by-product of the production of cottage cheese [17]. Since the product formed after the fermentation of the medium with sour whey by *G. geotrichum* was characterized by the highest intensity of aroma among all variants analyzed in previous studies, it is assumed that LAB metabolites can affect the metabolism of *G. geotrichum* [12]. In addition to affecting the physicochemical properties of fermented milk products and the metabolic transformation of microbial communities, they also affect their sensory properties, allowing the enrichment of the aroma mixture [18].

The aim of this study was to analyze the combined effect of the inoculation with LAB and *G. geotrichum* on the formation of aroma compounds during the fermentation of the sour whey medium [12]. This was achieved by analyzing the course of the culture and determining changes in the number of microorganisms; the concentration of amino acids, lactic acid, and selected key aroma compounds; and the pH of the medium. The post-fermentation product underwent sensomic analysis including the identification of key odorants by gas chromatography−olfactometry (GC−O), quantitation of the identified compounds using the stable isotope dilution assay (SIDA), and calculation of the aroma activity values (OAVs).

2. Results and Discussion

2.1. Cultivation Experiments

During the cultivation of LAB and *G. geotrichum* on the medium with sour whey, the pH values, the lactic acid concentration, the amounts of viable cells of analyzed microorganisms, the content of phenylacetaldehyde and 2-phenylethanol, and the amino acid profile were monitored. The monitoring of the culture course was aimed at analyzing the influence of the introduction of LAB pre-fermentation into the process of the production of aroma compositions by *G. geotrichum* on the sour whey medium.

2.1.1. Total Count of LAB and *G. geotrichum*, Lactic Acid Concentration, and pH during Bioreactor Culture

To verify the growth of LAB and *G. geotrichum* in one environment, the results of microbiological analyses were compared with the lactic acid concentration and pH during an 8-day culture. Figure 1 shows that both LAB and *G. geotrichum* added after 24 h were characterized by intensive growth on the sour whey medium.

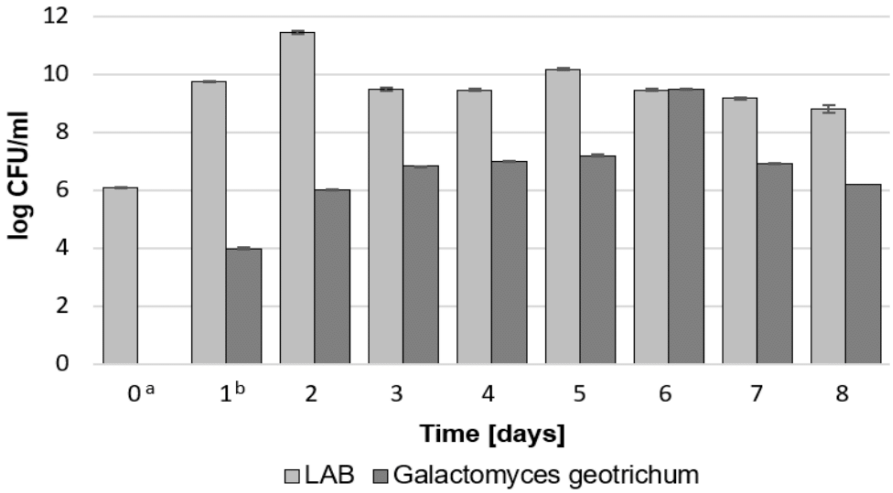

Figure 1. The concentration of LAB and *G. geotrichum* during 8-day culture on sour whey medium. [a] LAB inoculation. [b] *G. geotrichum* inoculation. Error bars represent ± SD from the replicates.

The highest concentration of viable LAB cells was observed on the 2nd day of culture in the bioreactor, and the highest concentration for *G. geotrichum* was observed on the 6th day (4 days from the inoculation of the medium). On the days above, LAB were counted at a level of 11.5 log CFU/mL and *G. geotrichum* were counted at 9.6 log CFU/mL. These dependencies are also observed in the results presented in Figure 2. The first 24 h of the LAB culture resulted in a noticeable decrease in the pH of the medium from 6.9 to 6.1 due to the production of lactic acid by these bacteria. The optimal environment for the growth of *G. geotrichum* is a pH in the range of 5.0–5.5, which was also confirmed in previous studies; therefore, before inoculation with the mold, the pH of the culture was adjusted to 5.0 using lactic acid [12,19]. Within the first 24 h of *G. geotrichum* growth in the presence of

LAB, the concentration of lactic acid decreased from 19.3 g/L to 16.0 g/L and then returned to its previous level. The observed decrease in the concentration of lactic acid did not affect the pH. This can be explained by the presence of sodium hydrogen phosphate in the medium, which has buffering properties [20]. In the following days, both the pH and the concentration of lactic acid remained constant. On the 5th day of culture, a decrease in the concentration of lactic acid and an increase in the pH of the medium were observed along with an increase in the concentration of LAB. Therefore, a slight increase in the pH of the environment above 5.0 had a positive effect on the development of LAB. A slight increase in the amount of LAB with the increasing pH of the environment was also observed during the tarhana fermentation with yeast and LAB [21]. With a gradual decrease in the number of LAB and *G. geotrichum* after 6 days of cultivation, a decrease in lactic acid concentration from 18.6 to 17.6 g/L and an increase in pH from 5.0 to 5.4 were observed. The obtained results can be explained by combining the lactic acid production capacity of LAB with the deacidification by the analyzed mold. *G. geotrichum* consume lactic acid and lactate, thus affecting the pH of the environment [22,23]. Lactic acid is not the primary source of carbon in the growth process of the analyzed molds, but it is possible that in the presence of a constant supply of this acid its consumption is intensified to counteract the decrease in pH of the environment. The literature data also reported the possibility of an increase in pH and lactic acid concentration during the cultivation of *G. geotrichum* (in the environment with LAB and separately). These differences may be due to the increase in the total count of certain LAB in co-cultures with *G. geotrichum* and different growth conditions, as well as differences due to the type of *G. geotrichum* strain [11,24,25].

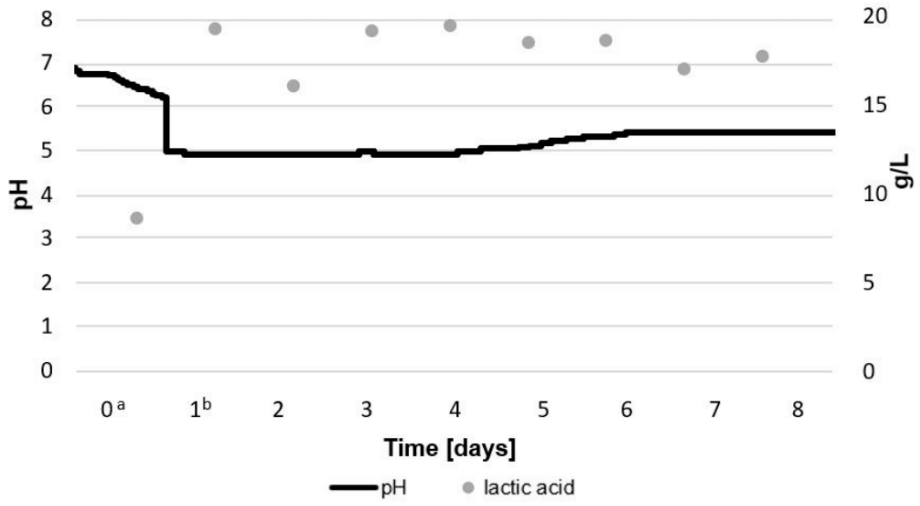

Figure 2. Lactic acid concentration and pH during an 8-day culture of LAB and *G. geotrichum* on acid whey medium. Lactic acid concentration was determined for samples taken every 24 h and pH was monitored every minute. [a] LAB inoculation. [b] *G. geotrichum* inoculation.

2.1.2. Content of Phenylacetaldehyde and 2-Phenylethanol during Cultivation of LAB and *G. geotrichum*

Phenylacetaldehyde with a honey-like odor and 2-phenylethanol with a rosy-like odor are aroma compounds produced during the transformation of L-phenylalanine in the Ehrlich pathway. This pathway involves the transamination of L-phenylalanine to phenylpyruvate, its decarboxylation to phenylacetaldehyde, and finally its reduction to 2-phenylethanol [26,27]. During the described transformations, both compounds are present in the fermented product, but in most cases a higher concentration of 2-phenylethanol is observed. However, the reverse ratio of these compounds provides a higher aromatizing power of the post-fermentation product due to the 60 times lower odor threshold (OT) of phenylacetaldehyde in water compared to that of 2-phenylethanol [26,27].

When analyzing the effect of LAB growth on the aroma produced by *G. geotrichum*, an experiment was conducted comparing the peak areas of these compounds on each day of the biotechnological process, and the results are presented in Figure 3. It can be seen that, from the detection of 2-phenylethanol in the medium after 3 days of cultivation, the ratio of phenylacetaldehyde to 2-phenylethanol remained higher than 1:1. With the increase in the area of the phenylacetaldehyde peaks with each subsequent day of cultivation, the values found for 2-phenylethanol also increased. The highest values for both compounds were observed on the last day of the biotechnological process.

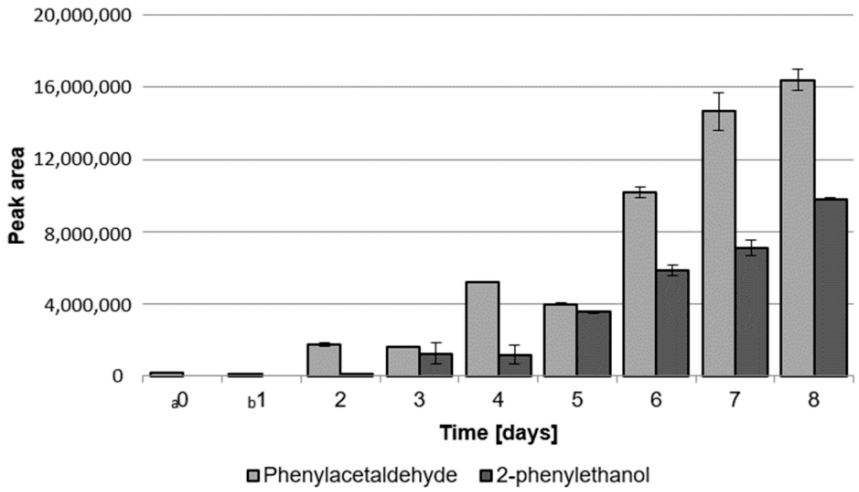

Figure 3. Peak areas of phenylacetaldehyde and 2-phenylethanol extracted from post-fermentation products obtained by biotransformation of sour whey medium by LAB and *G. geotrichum*. [a] LAB inoculation. [b] *G. geotrichum* inoculation. Error bars represent ± SD from the replicates.

2.1.3. Amino Acid Concentration during Biotransformation of Sour Whey Medium by LAB and *G. geotrichum*

It is well known that amino acids are some of the precursors of aroma compounds in biotransformation processes [28,29]. To identify the possible precursors of the aroma of the analyzed post-fermentation product, an analysis of the amino acid profile was carried out during the 8-day culture, and the results are presented in Figure 4.

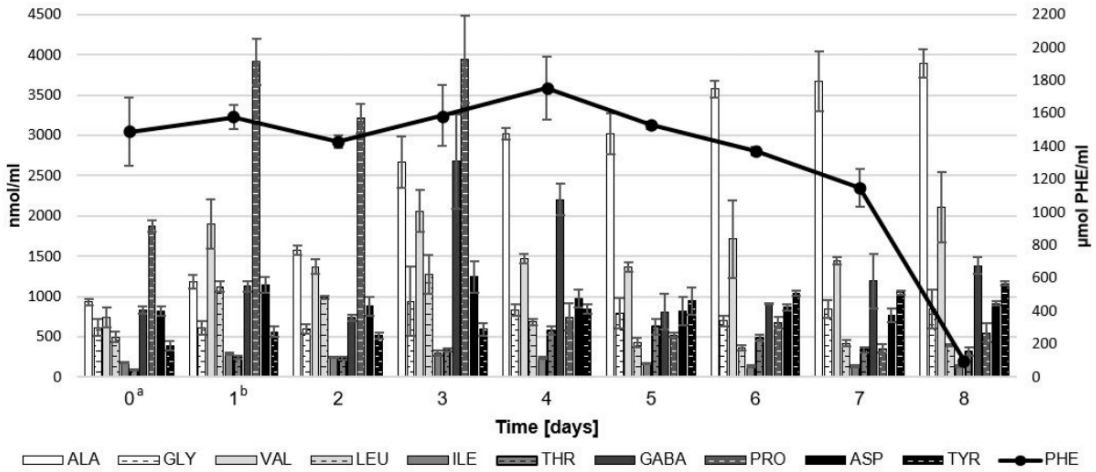

Figure 4. The concentration of free amino acids during 8-day fermentation of sour whey medium by LAB and *G. geotrichum*. [a] LAB inoculation. [b] *G. geotrichum* inoculation. Error bars represent ± SD from the replicates.

The analyzed medium and the fermentation product contained 11 free amino acids: alanine, glycine, valine, leucine, isoleucine, threonine, γ-aminobutyric acid (GABA), proline, aspartic acid, tyrosine, and L-phenylalanine. L-phenylalanine is a precursor of phenylacetaldehyde and 2-phenylethanol—compounds that were characterized by the highest OAVs in the aroma produced by *G. geotrichum* on substrates with by-products of the dairy industry [12,13,26]. It is an amino acid present in sour whey and is additionally supplemented with the substrate to ensure the possibility of its bioconversion to the highest possible concentration of honey and rose aroma compounds [30]. The results of the amino acid analysis showed that during the fermentation process of the medium with sour whey almost all L-phenylalanine was metabolized, with a radical decrease in its concentration on the last day of cultivation. A decrease in the concentration after 8 days of culture of LAB and *G. geotrichum* on sour whey medium was also observed for leucine, isoleucine, proline, and aspartic acid. Leucine and isoleucine are the precursors of fruity and malty aroma compounds, and the other amino acids may have been involved in cellular metabolism [29,31]. In the case of alanine, glycine, threonine, and tyrosine, an increase in their concentration was observed during cultivation. The concentrations of valine and GABA underwent alternating decreases and increases during the biotechnological process under study. The concentrations of valine and GABA underwent alternating decreases and increases during the biotechnological process under study. In the post-fermentation product, their concentration was finally higher than in the medium before fermentation. The periodic (e.g., in the case of L-phenylalanine) or total increase in the concentration of analyzed amino acids during the 8-day culture can be explained by the complex proteolytic and peptydolytic activity of the analyzed microorganisms, which enables the decomposition of proteins and peptides, enabling further transformations of amino acids [11,32,33].

2.2. Sensory Evaluation

The post-fermentation product obtained after culturing LAB and *G. geotrichum* on a medium with sour whey was subjected to sensory evaluation. The results presented in Figure 5 were compared to the odor profile of the product obtained by fermentation of the same medium under the same conditions with only *G. geotrichum* in previous studies [12].

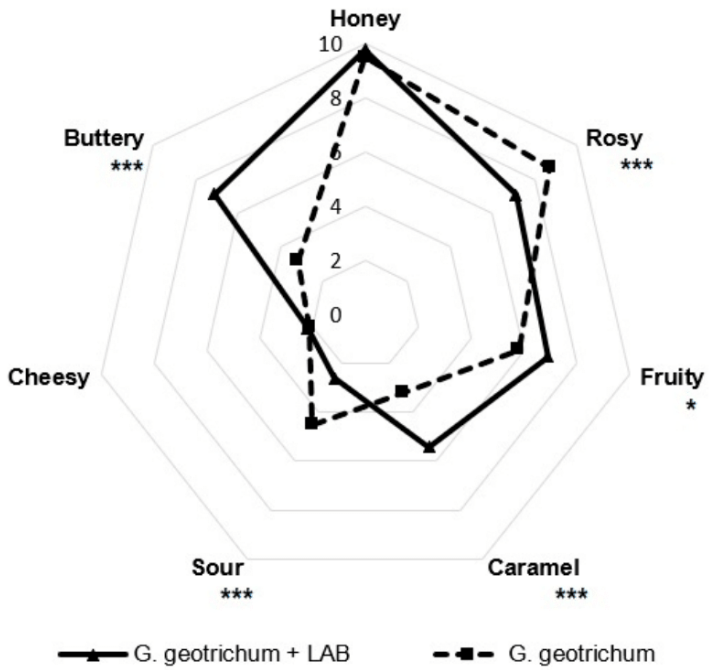

Figure 5. Sensory profiles of flavor mixtures after fermentation with LAB and *G. geotrichum* and only with *G. geotrichum* [12]. * $p < 0.05$; *** $p < 0.001$.

This experiment was aimed at analyzing the influence of introducing the pre-fermentation of the sour whey medium with LAB into the analyzed biotechnological process on the aroma profile of the obtained aroma composition. The product obtained after the fermentation of sour whey substrate by LAB and *G. geotrichum* was characterized by a high intensity of honey (9.8), rose (7.1), butter (7.1), fruit (6.9), and caramel (5.4) aroma notes.

The panelists pointed out a significant increase in the intensity of the buttery aroma (by 3.9) and noticeably more intense caramel (by 2.2) and fruity (by 1.1) aroma notes after introducing the initial fermentation with LAB to the analyzed biotechnological process. The honey aroma was again assessed as very strong, with a slight increase in perceptibility (by 0.3). In the case of the rose aroma, a decrease in its intensity (by 1.6) was observed in relation to the aroma produced by *G. geotrichum*, though it still remained a strongly perceptible note of the analyzed aroma. Cheese notes (2.2) and sour notes (2.6) were the least perceptible. In relation to the aroma composition obtained after the fermentation of sour whey exclusively by *G. geotrichum*, there was a decrease in cheese note intensity by 1.9.

2.3. Identification of Key Aroma Compounds in a Post-Fermentation Product Using GC−O Analysis and Calculation of OAVs

Samples collected after the bioreactor culture of LAB and *G. geotrichum* on the sour whey medium were subjected to SAFE extraction to perform a full characterization of the obtained aroma composition. During the GC-O analysis of the post-fermentation product, 18 aroma compounds were identified. The results of the quantification of the identified compounds and the OAVs are presented in Table 1.

Table 1. Aroma-active compounds identified in post-fermentation products from sour whey.

Compound [a]	Odor [b]	RI-DB 5 [c]	RI-Wax [c]	Concentration (µg/kg) [d]	OT in Water (µg/kg) [e]	OAV [f]
2,3-butanedione	buttery	670	991	3500	15	233
acetic acid	vinegar	691	1445	7080	99,000	<1
3-methyl-1-butanol	fruity	719	1204	1269	980	1.3
methyl butanoate	fruity	734	1052	375	60	6.2
2,3-butanediol	buttery	810	1583	15,464	150	103
butanoic acid	cheesy	836	1620	367	1000	<1
methyl hexanoate	fruity	923	1245	164	90	1.8
dimethyl sulfone	sulfuric	959	-	53,449 *	n.d.	-
ethyl hexanoate	pineapple	988	1229	17	1.2	14
hexanoic acid	sweaty	993	1224	2823	3000	<1
phenylacetaldehyde	honey	1080	1644	9440	5.2	1815
2-phenylethanol	rosy	1124	1920	5400	140	39
methyl octanoate	fruity	1131	1378	147	200	<1
ethyl octanoate	fruity	1194	1441	132	8.7	15
phenylacetic acid	honey	1260	2568	13,400	68	197
octanoic acid	musty	1268	1420	554	190	2.9
methyl decanoate	sweaty	1325	1590	<LOD [g]	4	-
vanillin	vanilla	1406	2590	56	53	1.1

[a] The compounds identified by comparison with reference compounds based on the following criteria: retention index (RI), mass spectra obtained by MS (EI), and odor quality at the sniffing port. [b] Odor perceived at the sniffing port. [c] Retention indices on DB-5 and SUPELCOWAX 10 columns. [d] Mean values based on three replicates with RSD value $\leq$ 11%. [e] OT: odor thresholds in water [34]. [f] OAV: odor activity values calculated by dividing the concentration of an analyte by its odor threshold value in water. [g] LOD—limit of detection. * Concentration estimated based on the internal standard [$^2H_8$] naphthalene.

An important element of the interpretation of the results of the identified aroma compounds in the analyzed composition are the OAVs. These are indicators calculated by dividing the concentration of a given compound by its OT value, determining the aroma compound activity. By comparing the OAVs of all identified aroma compounds in a given product, it is possible to determine their contribution to the final impression of the total aroma [35]. The OAVs calculated for aroma compounds in the product obtained after fermentation with LAB and *G. geotrichum* indicate that 12 of the 18 identified compounds have OAVs greater than 1.0, potentially contributing to the overall aroma of the fermentation product. In the analyzed aroma composition, five compounds were identified as having OAVs ranging from 0 to 1, five ranging from greater than 1 to 10, three ranging from greater than 10 to 100, three ranging from greater than 100 to 500, and one compound having OAV greater than 500. In the case of dimethyl sulfone, the OAV could not be calculated due to the unavailability of its OT value. Methyl decanoate, however, was identified at the stage of GC-O analysis, but the concentration of this compound turned out to be below the detection limit, which also made it impossible to calculate its OAV.

The obtained results indicate that the key odor compound shaping the aroma of the sour whey post-fermentation product with the highest OAV is phenylacetaldehyde (1815). At the same time, this compound was not characterized by the highest concentration among all identified. Such a high OAV is related to the very low OT (in water) value of phenylacetaldehyde, which is 4 μg/kg [34]. It should be noted that this value is 1.65 times lower than in the case of the aroma composition produced as a result of the fermentation of the sour whey medium exclusively by *G. geotrichum*; however, it did not affect the intensity of the honey aroma in the analyzed product. Moreover, the intensity of the honey note of the aroma increased by 0.3 [12]. This phenomenon is related to the perception of aroma. Although each aroma compound is characterized by a specific odor, the loss of their individual aroma descriptors is already observed in mixtures of four compounds. The compounds present in the mixture may have a synergistic effect and completely lose their individual fragrance notes, as well as condition the appearance of an aroma note unrelated to any of them. A complex aroma consisting of more than four aroma compounds (which is the case in most food products) should be considered not only as the sum of the aroma of individual compounds but mainly as a total effect [7,36,37]. Phenylacetaldehyde is formed from phenylalanine during the Ehrlich pathway. During bioconversion, phenylacetaldehyde can be transformed into 2-phenylethanol with a rosy aroma or phenylacetic acid with a honey aroma, which was also identified in the post-fermentation product with OAVs 39 and 197, respectively [26,29,38]. Phenylacetaldehyde, 2-phenyl ethanol, and phenylacetic acid were found in the tested variant in a ratio of 1.7:1:2.5. Comparing this result to previous studies with *G. geotrichum* without pre-fermentation with LAB, where the ratio was 1.7:1:0.2, a significant increase in the concentration of phenylacetic acid is observed while maintaining the current ratio of phenylacetaldehyde to 2-phenylethanol, which ensures an intense honey–rose aroma [12]. Similar observations were noted in Whetstine's research [36]: they concluded that phenylacetaldehyde combined with phenylacetic acid provides a more intense aroma in Cheddar cheese. Thus, a high concentration of phenylacetic acid may most likely significantly increase the intensity of the honey aroma of the final fermentation mixture.

In addition to compounds with a honey–rose aroma, the key odorants were also those with a buttery odor, which can also be observed in the aroma profile of the analyzed post-fermentation product. 2,3-butanedione was the second compound with the highest OAV (233), and 2,3-butanediol was fourth (103). The resulting concentration of 2,3-butanedione in the analyzed aroma composition is as much as 6.4 times higher than in previous studies involving *G. geotrichum* fermenting the sour whey medium. This correlates very clearly with sensory analysis. In combination with the identification of 2,3-butanediol at a concentration of 15,464 μg/kg, this resulted in a significant increase in the intensity of the pleasant buttery aroma in the analyzed composition. The presence of these compounds in such concentrations was undoubtedly the effect of LAB, which are characterized by the ability

to produce them. Compounds with a buttery aroma are some of the compounds shaping the aroma of yoghurt and other dairy products [33]. 2,3-butanedione is a compound formed during the transformation of sugars and lipids via various bacteria and has been reported as a key odorant in cheeses, such as fried cottage cheese, Cheddar, Camembert, and Lazur [8,36,39,40]. 2,3-butanediol, which is a reduced form of acetoin (also produced by LAB), has been identified as one of the key odorants in the traditional fermented milk product called "Lben" [41].

Among the compounds identified in the aroma mixture obtained after the fermentation of the sour whey medium by LAB and *G. geotrichum*, six compounds with a fruity aroma note were found. One of these compounds is 3-methyl-1-butanol, which is generated in the biotransformation of leucine [7,29]. During cultivation, a decrease in the concentration of this amino acid was observed, indicating a pathway for generating this aroma compound. 3-Methyl-1-butanol is responsible for the fruity smell of many fermented products, such as wine, cider, fermented meat products, and pumpernickel sourdough [42–45]. The concentration of this compound in the analyzed fermentation product was 95 times lower than in the product after the fermentation of the sour whey medium only by *G. geotrichum* [12]. Nevertheless, an increase in the intensity of the fruity aroma was observed in the composition after fermentation by LAB and *G. geotrichum*. This draws attention to the possibility of intensifying the fruity aroma by combining various esters and 3-methyl-1-butanol in one mixture. Among the identified esters, the highest OAVs were found in ethyl octanoate (15) and ethyl hexanoate (14), which are also some of the eight esters included in the aroma of yoghurt as a result of fermentation by LAB and key odorants in alcoholic beverages [7,33].

Another key odorant is vanillin with an OAV of 1.1, which has not yet been found in the aroma produced by *G. geotrichum*. Vanillin, widely used in the food and cosmetic flavor industry due to its pleasant vanilla aroma, can be formed biotechnologically by some *Lactobacillus* sp. by the transformation of ferulic acid [28,46]. Four acids were also identified in the fermentation product, of which only octanoic acid with a musty odor was characterized by an OAV > 1. It is worth noting that a 35 and 50 times decrease in the concentration of acetic acid and butanoic acid was observed, respectively, in relation to the culture without LAB pre-fermentation [12]. The decrease in the concentration of these acids reduces unpleasant odors, such as vinegar-like and cheese-like odors, which result from their high concentrations in the product. The last key odorant in the product resulting from the fermentation of the sour whey medium by LAB and *G. geotrichum* is dimethyl sulfone. This compound was found in the analyzed product in the highest concentration (53,449 µg/kg) among all identified compounds, but no sulfur note of aroma was found for it. The possibility of its synthesis by biotechnology in food products has not been demonstrated so far, but it has been described as a new marine biogenic emission. Therefore, the most probable pathway of dimethyl sulfone production is the described oxidation of dimethyl sulfide by OH radicals [47]. Dimethyl sulfone should be considered in terms of the element of the aroma composition affecting its overall perception.

It should be noted that four of the identified aroma compounds (2,3-butanedione, acetic acid, butanoic acid, and vanillin) belong to the group referred to as "generalists", constituting the key aroma compounds of more than 25% of food products, while seven compounds (phenylacetaldehyde, 2-phenylethanol, phenylacetic acid, ethyl hexanoate, ethyl octanoate, hexanoic acid, and 3-methyl-1-butanol) are included in the "intermediaries" group, shaping the aroma of from 5 to 25% of food products [7].

The influence of mold *G. geotrichum* on LAB is described in the literature. In Chaves-López's [11] research, it was observed that *G. geotrichum*, when present in an environment with LAB, increased their viability. This has the effect of compensating for the decrease in the lactic acid production capacity of the LAB with the duration of the culture. The obtained results show that the interactions between *G. geotrichum* and LAB are mutual. The implementation of LAB pre-fermentation to the production of aroma compounds by *G. geotrichum* on a sour whey medium increased the amount of aroma-active odorants identified in the obtained mixture. Changing the composition of the fermentation product

resulted in an increase in the intensity of pleasant fragrance notes (honey, rosy, caramel) and a reduction in irritating odors by their lower concentration.

3. Materials and Methods

3.1. Chemicals

Spray-dried sour whey was obtained from Laktopol (Suwałki, Poland). L-Phenylalanine, lactic acid, sodium sulfate, dichloromethane, diethyl ether, and Man Rogosa Sharpe (MRS) agar were purchased from Sigma-Aldrich (Poznań, Poland). Yeast extract and a medium with chloramphenicol were obtained from BTL (Łódź, Poland). Citric acid, $Na_2HPO_4 \cdot 2H_2O$, and $MgCl_2$ were obtained from POCH (Gliwice, Poland). Inulin was purchased from Hortimex Plus (Konin, Poland). An EZ: Faast™ Kit for Free (Physiological) Amino Acids was obtained from Phenomenex (Aschaffenburg, Germany). The following reference aroma compounds were purchased from Sigma-Aldrich (Poznań, Poland): 2,3-butanedione, acetic acid, 3-methyl-1-butanol, methyl butanoate, 2,3-butanediol, butanoic acid, methyl hexanoate, dimethyl sulfone, ethyl hexanoate, hexanoic acid, phenylacetaldehyde, 2-phenylethanol, methyl octanoate, ethyl octanoate, phenylacetic acid, octanoic acid, methyl decanoate, vanillin, and lactic acid. The following stable isotopes were obtained from AromaLAB (Freising, Germany): [$^{13}C_4$] 2,3-butanedione, [$^{13}C_1$] acetic acid, [$^{2}H_2$] 3-methyl-1-butanol, [$^{2}H_9$] ethyl 3-methylbutanoate, [$^{2}H_5$] phenylacetaldehyde, [$^{2}H_5$] 2-phenylethanol, [$^{2}H_3$] vanillin, and [$^{2}H_8$] naphthalene.

3.2. Microorganisms

The strain 32 *G. geotrichum* came from the collection of the Food Volatilomics and Sensomics Group, Poznań University of Life Sciences. The analyzed mold strain was isolated from Wielkopolski fried cottage cheese produced in the vicinity of Poznań in Poland during the ripening stage. The *G. geotrichum* strain was protected by freeze-drying. The preparation of the *G. geotrichum* strain for this study followed the methodology described in our previous studies [12,13].

A lyophilized starter culture of heterofermentative LAB (*Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *lactis* biovar *diacetilactis*, *Leuconostoc mesenteroides* subsp. *cremoris*) for the production of cottage cheese was obtained from Biochem Srl (Montelibretti, Italy).

3.3. Bioreactor Culture

The cultivation of LAB and *G. geotrichum* was carried out in a 2.3 L Labfors 5 bioreactor (Infors HT, Bottmingen, Switzerland) with a working volume of 2 L. The microorganisms were grown in a medium containing, per liter (modified, based on Grygier et al. [48]), 22.8 g of $Na_2HPO_4 \cdot 2H_2O$, 0.5 g of $MgCl_2$, 0.17 g of yeast extract, 60 g of sucrose, 130 g of spray-dried sour whey, and 21 g of L-phenylalanine. Sucrose, sour whey, and L-phenylalanine were added to the sterilized medium after exposure to UV radiation for 30 min due to the risk of changes in the aroma profile of the post-fermentation product due to the effect of high temperatures on these compounds. The medium was inoculated with a cottage cheese starter culture according to the producer's recommendations (6×10^9 CFU) to best reflect the natural growth environment of *G. geotrichum* isolated from fried cottage cheese. The pre-fermentation of the sour whey medium by LAB was carried out for 24 h at 30 °C with stirring at a speed of 150 rpm. After initial fermentation, the pH of the medium was adjusted to 5.0 with lactic acid and inoculated with 3.4×10^7 CFU of lyophilized *G. geotrichum* mold. The cultivation was carried out for 7 days at 30 °C with stirring at 150 rpm and aeration to 1.5 vvm, with the inlet air sterilized by filtration. The pH value was not adjusted during the culture but was constantly monitored. A blank test was also performed using the same culture conditions without the inoculation of sour whey medium with LAB and *G. geotrichum.*

3.4. Cultivation Experiments

3.4.1. Total Count of LAB and *G. geotrichum*

In order to determine the number of *G. geotrichum* colony-forming units (CFUs), serial dilutions of the culture were prepared and plated onto plates with a solid medium with chloramphenicol, which were incubated for 72 h at 30 °C under aerobic conditions. The determination of the number of LAB CFUs was carried out in an analogous way, using the MRS agar and incubation for 48 h at 30 °C under anaerobic conditions. After the incubation of the agar plates, the CFUs were counted and the results were converted to CFUs per milliliter. The experiment was performed on each culture day in triplicate.

3.4.2. Determination of Lactic Acid Concentration

Samples taken during bioreactor culture of LAB and *G. geotrichum* were diluted with distilled water and centrifuged for 10 min at 13,000× *g* using a Biofuge Primo R centrifuge (Heraeus, Hanau, Germany). The supernatant obtained was passed through 0.45 µm mixed cellulose ester syringe filters (Lab Logistics Group GmbH, Meckenheim, Germany). The obtained samples underwent HPLC analysis to identify and quantify the lactic acid, using an Agilent 1200 series chromatograph with an autosampler and refractive index detector (RID) (Agilent Technologies, Santa Clara, CA, USA). The apparatus was equipped with a REZEX-ROA column (300 mm × 7.8 mm, Phenomenex, Aschaffenburg, Germany). The samples (10 µL) were injected into the HPLC column using the splitless mode. Lactic acid was analyzed at 40 °C with 0.005 N H_2SO_4 as a mobile phase at a flow rate of 0.6 mL/min. Lactic acid was identified by comparison with a reference compound based on the retention index. Quantitation was performed with an external standard curve. The analysis was carried out on samples taken during each day of culture in the bioreactor in order to study changes in lactic acid concentration during its duration.

3.4.3. Analysis of the Amino Acid Profile

Samples taken during the cultivation of LAB and *G. geotrichum* on a whey medium were diluted 1:1 with distilled water and centrifuged at 4000 rpm for 15 min using a MPW-223e centrifuge (MPW MED. INSTRUMENTS, Warsaw Poland). The supernatant obtained was passed through 0.45 µm cellulose acetate syringe filters (Lab Logistics Group GmbH, Meckenheim, Germany). The sample obtained was directly analyzed using the EZ: Faast™ Kit for Free (Physiological) Amino Acids (Phenomenex, Aschaffenburg, Germany) according to the producer's recommendations. The analytical procedure involves the solid-phase extraction of 50 µL of extract, followed by derivatization and liquid–liquid extraction. Amino acids were separated in the Agilent 7890A gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) equipped with an autosampler G45134, using the Agilent 5975C Mass Selective Detector. Derivatized amino acids were separated in the ZB-AAA EZ Faast™ capillary column (10 m × 0.25 mm, Phenomenex, Aschaffenburg, Germany). The carrier gas was helium (1.5 mL/min). The samples (2 µL) were injected in the split mode set as 1:50 and 1:5 for phenylalanine and other amino acids, respectively. The oven temperature was initially set at 110 °C and then increased to 320 °C (30 °C/min). The injector and ion source temperatures were 250 °C and 240 °C, respectively. Mass spectra were obtained by electron ionization (EI) over the range of 35–550 m/z. The electronic impact energy was 70 eV. The amino acids were identified and quantified using standards for each amino acid and normalized in relation to the internal standard (norvaline). The analysis was carried out on samples taken during each day of the bioreactor culture in order to examine changes in the amino acid profile during its duration.

3.5. Sensory Evaluation

The sour whey medium after fermentation with LAB and *G. geotrichum* was subjected to descriptive sensory evaluation by ten experienced panelists. In this experiment, seven aroma descriptors (honey, caramel, rosy, fruity, cheesy, sour, and buttery) were assessed in triplicate during separate profile sessions. The analyzed characteristics were selected from

the basic flavor descriptive language (Givaudan Roure Flavor [49]) and applied in previous studies. Sensory evaluation was performed by presenting to the panelists a 20 g sample of the post-fermentation product in 100 mL glass containers at room temperature. Panelists scored each odor descriptor on a 10 cm linear scale, with the beginning labeled "none" and the end labeled "very strong". The results have been converted to numerical values. The data obtained for each descriptor were averaged and subjected to t-test analysis.

3.6. Extraction of Odor-Active Compounds Using the Solvent-Assisted Flavor Evaporation (SAFE) Method

The aroma mixture obtained after the bioreactor culture of LAB and *G. geotrichum* on sour whey medium underwent extraction using the SAFE method described by Engel et al. [50] in order to isolate the odor-active compounds. The samples (50 g) were mixed with dichloromethane (100 mL) and spiked with the internal standard [$^2H_8$] naphthalene (25 µg), then they were shaken for 2 h on a horizontal shaker. After the extraction process, the samples were subjected to the isolation of volatile compounds using the SAFE method. The obtained extracts were dried over anhydrous sodium sulfate and concentrated using a Kuderna Danish concentrator (Sigma-Aldrich, Poznań, Poland) to a volume of approximately 500 µL.

3.7. GC-O and GC-GC Analysis

3.7.1. Gas Chromatography–Olfactometry (GC-O)

The identification of odor-active compounds was carried out by subjecting extracts obtained by the SAFE method to GC-O analysis. The identification was performed using an HP 5890 chromatograph (Hewlett- Packard, Wilmington, DE, USA) with two columns of different polarities: SPB-5 (30 m × 0.53 mm × 1.5 µm) and SUPELCOWAX 10 (30 m × 0.53 mm × 1 µm) (Supelco, Bellefonte, PA, USA). The effluent was divided between the olfactometry port with humidified air as a makeup gas and a flame ionization port using a Y splitter in GC. The operating conditions for the SPB-5 column were as follows: an initial oven temperature of 40 °C (1 min) raised at 6 °C/min to 180 °C and at 20 °C/min to 280 °C. For the SUPELCOWAX 10 column, the operating conditions were as follows: an initial oven temperature of 40 °C (2 min) raised to 240 °C at a 6 °C/min rate and held for 2 min isothermally. The injection of the SAFE extract (2 µL) into a GC column was performed in a splitless mode. In order to detect active regions and specific notes of selected volatiles, three panelists sniffed the GC-O effluent. For all peaks and flavor descriptors with specific retention times, retention indices were calculated in order to compare the results with those obtained by GC−MS and literature data. For each compound, the retention indices (RIs) were calculated using a homologous series of C7−C24 n-alkanes.

3.7.2. Comprehensive Two-Dimensional Gas Chromatography (GC-GC)

For the identification and quantitation of the aroma compounds, samples were run on a comprehensive gas chromatography–mass spectrometry system—GCxGC-ToF-MS (Pegasus 4, LECO, St. Joseph, MI). The GC was equipped with an SLB-5MS column (30 m × 0.25 mm × 0.25 µm) and SPB-50 (1.2 m × 0.25 mm × 0.25 µm) as a second column. For two-dimensional analysis modulation (liquid N modulator by ZOEX), the time was optimized and set at 4 s, and mass spectra were collected at a rate of 150 scans/s. The transfer line was heated up to 260 °C, and the ion source was heated to 220 °C, respectively. For all the volatiles, identification was performed by a comparison of mass spectra, retention indices (RI) on two columns of different polarities to those of standard compounds, the National Institute of Standards and Technology (NIST) 09 Mass Spectral Library, and literature data.

3.8. Quantitation by Stable Isotope Dilution Assays (SIDAs)

For all of the compounds identified in the GC-O experiment, stock internal standards of the labeled isotopes were prepared in diethyl ether and added to the analyzed samples

in a concentration similar to that of the relevant analyte. The volatiles were analyzed by GCxGC-ToF-MS monitoring the intensities of the respective ions given in Table 2. For all the volatiles, response factors were calculated in the standard mixture of labeled and unlabeled compounds at a known concentration of 500 ppb. The concentrations in the samples were calculated from the peak area of the analyte and its corresponding internal labeled standard obtained for selected ions. Finally, odor activity values (OAVs) were calculated by dividing the concentration of a given analyte by its odor threshold (OT) determined in water.

Table 2. Labeled standards and quantitation ions used for SIDA (stable isotope dilution assay) concentration calculations of 13 key odorants present in the post-fermentation products.

Compound [a]	Quant. Ions (*m*/*z*) [b] (*m*/*z*) [b]	Labeled Standards [c]	Ion IS (*m*/*z*) [d]
2,3-butanedione	86	[$^{13}C_4$] 2,3-butanedione	90
acetic acid	60	[$^{2}H_3$] acetic acid	63
3-methyl-1-butanol	70	[$^{2}H_2$] 3-methyl-1-butanol	72
methyl butanoate	102	[$^{2}H_9$] ethyl 3-methylbutanoate	139
2,3-butanediol	90	[$^{13}C_4$] 2,3-butanedione	90
butanoic acid	73	[$^{2}H_7$] butanoic acid	77
methyl hexanoate	130	[$^{2}H_9$] ethyl 3-methylbutanoate	139
dimethyl sulfone	94	[$^{2}H_8$] naphthalene	131
ethyl hexanoate	144	[$^{2}H_9$] ethyl 3-methylbutanoate	139
hexanoic acid	116	[$^{2}H_7$] butanoic acid	77
phenylacetaldehyde	120	[$^{2}H_5$] phenylacetaldehyde	125
2-phenylethanol	122	[$^{2}H_5$] 2-phenylethanol	127
methyl octanoate	158	[$^{2}H_9$] ethyl 3-methylbutanoate	139
ethyl octanoate	172	[$^{2}H_9$] ethyl 3-methylbutanoate	139
phenylacetic acid	136	[$^{2}H_5$] 2-phenylethanol	127
octanoic acid	122	[$^{2}H_7$] butanoic acid	77
methyl decanoate	186	[$^{2}H_9$] ethyl 3-methylbutanoate	139
vanillin	152	[$^{2}H_3$] vanillin	155

[a] Quantified compounds; [b] ions used for quantitation of analytes; [c] corresponding labeled standards used for quantitation; [d] ions of internal standards (labeled isotopes) used for quantitation.

4. Conclusions

The results of this study indicated that in addition to the known effect of *G. geotrichum* on LAB in one environment the dependence is also reversed. LAB are part of the microflora of the environment where the mold *G. geotrichum* is naturally present. The initial fermentation of sour whey by LAB reproduces the natural conditions for the development of the analyzed molds by the presence of the effects of their metabolism. LAB's ability to produce lactic acid adjusted the pH of the medium to the growth of *G. geotrichum*, reducing the amount of lactic acid added for its regulation. Moreover, lactic acid synthesized throughout the culture was an additional source of carbon for the analyzed mold. Low molecular weight metabolites resulting from LAB growth can affect *G. geotrichum* by imitating their natural development environment.

The combined effect of the LAB and *G. geotrichum* fermentation of sour whey increased the amount of odor-active compounds in the aroma composition. The GC-O identified 18 aroma compounds. The OAV calculation allowed for the identification of 12 aroma-active compounds among them. The key odorant shaping the aroma of the analyzed composition is phenylacetaldehyde with a honey aroma note. The next compounds with the highest OAVs were 2,3-butanedione (buttery), phenylacetic acid (honey), 2,3-butanediol (buttery), 2-phenylethanol (rosy), ethyl octanoate (fruity), and ethyl hexanoate (fruity). The introduction of LAB pre-fermentation into the bioconversion process by *G. geotrichum* also resulted in changes in the concentration of aroma compounds identified in recent studies. As a result, the intensity of pleasant aroma notes (honey, buttery, rosy, caramel) increased and the irritating aroma notes of acids (vinegar-like, cheesy) were reduced.

The obtained results provide basic knowledge on the possibility of intensifying the aroma produced by *G. geotrichum* mold. The effect of the combined fermentation of sour whey by LAB and *G. geotrichum* provides an aroma composition with an intense, honey–buttery aroma. It gives the opportunity to enrich the aroma of not only dairy industry products but also, for example, the products of the bakery, confectionery, and brewing industries.

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