UNIVERSIDADE FEDERAL DO PARANÁ

BRUNA LEAL MASKE

PREDICTING MICROBIOMES, VIRAL INTERACTIONS, AND FUNCTIONAL GENES IN THE PRODUCTION OF FUNCTIONAL FOODS: AN APPLICABLE MODEL USING NATURAL VINEGAR FERMENTATION

CURITIBA

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Tese apresentada ao curso de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia, Setor de Tecnologia, Universidade Federal do Paraná, como requisito parcial à obtenção do título de doutor em Engenharia de Bioprocessos e Biotecnologia.

Orientador: Prof. Dr. Gilberto Vinícius de Melo Pereira

Coorientador: Prof. Dr. Carlos Ricardo Soccol

CURITIBA

2025

DADOS INTERNACIONAIS DE CATALOGAÇÃO NA PUBLICAÇÃO (CIP)
UNIVERSIDADE FEDERAL DO PARANÁ
SISTEMA DE BIBLIOTECAS – BIBLIOTECA CIÊNCIA E TECNOLOGIA

Maske, Bruna Leal
Predicting microbiomes, viral interactions, and functional genes in the production of functional foods: an applicable model using natural vinegar fermentation. / Bruna Leal Maske. – Curitiba, 2025.
1 recurso on-line : PDF.

Tese (Doutorado) - Universidade Federal do Paraná, Setor de Tecnologia, Programa de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia.

Orientador: Prof. Dr. Gilberto Vinícius de Melo Pereira
Coorientador: Prof. Dr. Carlos Ricardo Soccol

1. Bactérias do ácido lático. 2. Probióticos. 3. Alimentos funcionais. 4. Metagenômica. I. Universidade Federal do Paraná. II. Programa de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia. III. Pereira, Gilberto Vinícius de Melo. IV. Soccol, Carlos Ricardo. V. Título.

Bibliotecária: Roseny Rivelini Morciani CRB-9/1585

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ATA Nº195

ATA DE SESSÃO PÚBLICA DE DEFESA DE DOUTORADO PARA A OBTENÇÃO DO GRAU DE DOUTORA EM ENGENHARIA DE BIOPROCESSOS E BIOTECNOLOGIA

No dia vinte e oito de fevereiro de dois mil e vinte e cinco às 09:00 horas, na sala sala 03, Prédio de Engenharia de Bioprocessos e Biotecnologia, foram instaladas as atividades pertinentes ao rito de defesa de tese da doutoranda **BRUNA LEAL MASKE**, intitulada: **PREDICTING MICROBIOMES, VIRAL INTERACTIONS, AND FUNCTIONAL GENES IN THEPRODUCTION OF FUNCTIONAL FOODS: AN APPLICABLE MODEL USING NATURAL VINEGAR FERMENTATION**, sob orientação do Prof. Dr. GILBERTO VINICIUS DE MELO PEREIRA. A Banca Examinadora, designada pelo Colegiado do Programa de Pós-Graduação ENGENHARIA DE BIOPROCESSOS E BIOTECNOLOGIA da Universidade Federal do Paraná, foi constituída pelos seguintes Membros: GILBERTO VINICIUS DE MELO PEREIRA (UNIVERSIDADE FEDERAL DO PARANÁ), JULIO CESAR DE CARVALHO (UNIVERSIDADE FEDERAL DO PARANÁ), ADRIANE BIANCHI PEDRONI MEDEIROS (UNIVERSIDADE FEDERAL DO PARANÁ), JOSE GUILHERME PRADO MARTIN (UNIVERSIDADE FEDERAL DE VIÇOSA), DÃO PEDRO DE CARVALHO NETO (INSTITUTO FEDERAL DO PARANÁ). A presidência iniciou os ritos definidos pelo Colegiado do Programa e, após exarados os pareceres dos membros do comitê examinador e da respectiva contra argumentação, ocorreu a leitura do parecer final da banca examinadora, que decidiu pela APROVAÇÃO. Este resultado deverá ser homologado pelo Colegiado do programa, mediante o atendimento de todas as indicações e correções solicitadas pela banca dentro dos prazos regimentais definidos pelo programa. A outorga de título de doutora está condicionada ao atendimento de todos os requisitos e prazos determinados no regimento do Programa de Pós-Graduação. Nada mais havendo a tratar a presidência deu por encerrada a sessão, da qual eu, GILBERTO VINICIUS DE MELO PEREIRA, lavrei a presente ata, que vai assinada por mim e pelos demais membros da Comissão Examinadora.

CURITIBA, 28 de Fevereiro de 2025.

Assinatura Eletrônica
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JOSE GUILHERME PRADO MARTIN
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TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação ENGENHARIA DE BIOPROCESSOS E BIOTECNOLOGIA da Universidade Federal do Paraná foram convocados para realizar a arguição da tese de Doutorado de **BRUNA LEAL MASKE**, intitulada: **PREDICTING MICROBIOMES, VIRAL INTERACTIONS, AND FUNCTIONAL GENES IN THEPRODUCTION OF FUNCTIONAL FOODS: AN APPLICABLE MODEL USING NATURAL VINEGAR FERMENTATION**, sob orientação do Prof. Dr. GILBERTO VINICIUS DE MELO PEREIRA, que após terem inquirido a aluna e realizada a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

A outorga do título de doutora está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

CURITIBA, 28 de Fevereiro de 2025.

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Dedico este trabalho à minha família, Beatriz,
Solange e Irineu.

AGRADECIMENTOS

A Deus, pela dádiva da vida. Viver como um ser consciente na Terra é um presente e tento aproveitar sempre ao máximo;

À Universidade Federal do Paraná e ao Curso de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia (PPGEBB) pela oportunidade concedida para a realização do doutorado;

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) e ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) pelo aporte financeiro para realização do estudo;

Ao meu orientador, Prof. Dr. Gilberto Vinícius de Melo Pereira, pela orientação cuidadosa, dedicação, disponibilidade e paciência, bem como pelas valiosas oportunidades oferecidas para participar na produção científica; agradeço especialmente por acreditar em mim e me apoiar, mesmo quando iniciei um novo trabalho no SENAI;

Aos meus pais, Solange e Irineu, que nunca mediram esforços para a realização de todos os meus sonhos. Sou imensamente grata por toda força, presença, parceria, carinho, compreensão e confiança. Sem vocês nada disso teria sido possível;

À minha irmã Beatriz, por ser sempre minha alma gêmea e meu porto seguro. Você sempre me mostra o ponto de luz quando tudo parece escuro; Feliz por existir na Terra no mesmo tempo que você;

Às famílias do Rio de Janeiro e Rio Grande do Sul, que, mesmo distantes, sempre estiveram no meu coração;

Aos colegas do PPGEBB, que se tornaram amigos ao longo desta jornada, pelo apoio nas horas difíceis, pelos momentos de descontração compartilhados e, em especial, ao Alexander e Ignácio, cuja ajuda foi importante para a realização deste trabalho;

A todos meus amigos pelo companheirismo e parceria que tornaram meus dias mais divertidos nessa jornada.

Obrigada!

“Quando a Lua apareceu
Ninguém sonhava mais do que eu
Já era tarde, mas a noite é uma criança; distraída

Depois que eu envelhecer
Ninguém precisa mais me dizer
Como é estranho ser humano nessas horas
De partida

Ah, é o fim da picada
Depois da estrada começa uma grande avenida
No fim da avenida
Existe uma chance, uma sorte, uma nova saída

Qual é a moral?
Qual vai ser o final dessa história?
Eu não tenho nada pra dizer; por isso digo
Eu não tenho muito o que perder; por isso jogo
Eu não tenho hora pra morrer; por isso sonho
Ah, são coisas da vida…”

Rita Lee, “Coisas da vida”

RESUMO

Os alimentos fermentados são consumidos há séculos em diversas culturas, oferecendo sabores únicos, maior vida útil e benefícios à saúde. Recentemente, o interesse por esses produtos cresceu devido ao seu potencial como alimentos funcionais, especialmente por promoverem a saúde intestinal e fornecerem compostos bioativos. Tais propriedades derivam de comunidades microbianas complexas, compostas por bactérias, leveduras e, mais recentemente, reconhecidamente, vírus. Com o advento das tecnologias de sequenciamento de nova geração (*Next Generation Sequencing* – NGS), tornou-se possível explorar a diversidade, a dinâmica e as funções desses microrganismos e seus genes, revelando novas espécies, vias metabólicas e interações vírus-hospedeiro relevantes para as características funcionais dos alimentos fermentados. Esta tese, estruturada em seis capítulos, integra revisões e estudos experimentais. Os dois primeiros capítulos abordam o papel das bactérias do ácido lático (BAL), tanto na fermentação de vinagre quanto na produção de enzimas funcionais. As BAL têm papel essencial nos estágios iniciais da fermentação, produzindo ácido lático e metabólitos bioativos. Identificaram-se lacunas na compreensão da viabilidade das BAL na produção de vinagre, e ressaltou-se o potencial do vinagre como fonte de produtos pós-bióticos. Além disso, as enzimas derivadas de lactobacilos foram destacadas por sua importância na digestão, na biodisponibilidade de nutrientes e em aplicações industriais, como a redução da intolerância à lactose. O terceiro capítulo apresenta um estudo metagenômico sobre a fermentação natural de vinagre de maçã. A análise revelou espécies microbianas-chave, como *Acetobacter pasteurianus*, *Komagataeibacter europaeus*, *Lactiplantibacillus plantarum* e *Saccharomyces cerevisiae*. Observou-se uma sucessão microbiana: BAL dominaram os estágios iniciais, seguidas pelas bactérias do ácido acético (BAA), responsáveis pela acidez e aroma do vinagre. Correlações entre grupos microbianos e compostos como ácido acético e acetato de etila demonstraram a importância do co-metabolismo na qualidade sensorial do produto. Os capítulos quatro e cinco exploram o papel dos vírus, especialmente bacteriófagos, nos alimentos fermentados. Eles modulam comunidades bacterianas e afetam diretamente os processos fermentativos. Também foram discutidas as interações entre leveduras e vírus, como as leveduras *killer*, com destaque para o viroma como ferramenta para melhorar a segurança, a qualidade e a biotecnologia alimentar. No sexto capítulo, foi empregada metagenômica *shotgun* para avaliar a dinâmica microbiana e viral durante três meses de fermentação de vinagre de

maçã. A análise revelou vias metabólicas relevantes e evidenciou a atuação de espécies como *A. ghanensis*, *Leuc. pseudomesenteroides* e *S. cerevisiae*, além do papel regulatório dos bacteriófagos sobre bactérias deteriorantes. Em conclusão, esta tese demonstra como abordagens metagenômicas permitem desvendar a complexidade microbiana e viral dos alimentos fermentados. Ao integrar microbiomas, viromas e genes funcionais, a pesquisa oferece novas perspectivas para o controle e aprimoramento da fermentação, qualidade dos produtos e desenvolvimento de alimentos funcionais inovadores.

Palavras-chave: Bactérias do Ácido Lático; Probióticos; Metagenômica; Metabolômica; Viroma; Alimentos funcionais.

ABSTRACT

Fermented foods have been consumed for centuries across diverse cultures, offering unique flavors, extended shelf life, and significant health benefits. In recent years, global interest in these products has increased due to their potential as functional foods, particularly for promoting gut health and delivering bioactive compounds. These properties stem from complex microbial communities composed of bacteria, yeasts, and, more recently acknowledged, viruses. With the advent of next-generation sequencing (NGS) technologies, it has become possible to explore the diversity, dynamics, and functions of these microorganisms and their associated genes, revealing novel species, metabolic pathways, and virus-host interactions that contribute to the functional characteristics of fermented foods. This thesis, structured in six chapters, integrates literature reviews and experimental studies. The first two chapters focus on the role of lactic acid bacteria (LAB) in vinegar fermentation and in the production of functional enzymes. LAB plays a crucial role in the early stages of fermentation, producing lactic acid and bioactive metabolites. Critical knowledge gaps were identified regarding LAB viability during vinegar production, and vinegar was highlighted as a potential source of postbiotic products. Additionally, enzymes derived from lactobacilli were discussed for their relevance in human digestion, nutrient bioavailability, and industrial applications such as reducing lactose intolerance and improving protein hydrolysis in food processing. The third chapter presents a metagenomic study of natural apple vinegar fermentation. The analysis revealed key microbial species such as *Acetobacter pasteurianus*, *Komagataeibacter europaeus*, *Lactiplantibacillus plantarum*, and *Saccharomyces cerevisiae*. Distinct patterns of microbial succession were observed: LAB dominated the initial stages, followed by acetic acid bacteria (AAB), which were primarily responsible for vinegar's acidity and aroma. Correlations between microbial groups and compounds such as acetic acid and ethyl acetate underscored the importance of co-metabolism in shaping the product's sensory quality. Chapters four and five explore the roles of viruses—especially bacteriophages—in fermented foods. These viruses modulate bacterial communities and directly influence fermentation processes. Interactions between yeasts and viruses, including the ecological roles of killer yeasts, are also discussed. These chapters emphasize the importance of virome studies for food safety and quality, advances in detection methods, and the potential of viral systems to enhance fermentation outcomes and optimize food biotechnology. The sixth chapter employs

shotgun metagenomics to assess microbial and viral dynamics during a three-month apple vinegar fermentation process. The study revealed key metabolic pathways related to carbohydrate and amino acid metabolism, energy production, and glycan biosynthesis. Specific enzymatic activities from *A. ghanensis*, *Leuc. pseudomesenteroides*, and *S. cerevisiae* were found to significantly shape the vinegar's sensory and biofunctional profile, while bacteriophages played a regulatory role by controlling spoilage bacteria. In conclusion, this thesis demonstrates how advanced sequencing technologies can unravel the complexity of microbial and viral communities in fermented foods. By linking microbiomes, viromes, and functional gene networks, the research highlights opportunities to improve fermentation processes, enhance product quality, and develop innovative functional foods.

Keywords: Lactic Acid Bacteria; Probiotic; Metagenomic; Metabolomic; Virome; Functional foods.

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SUMMARY

INTRODUCTION

The global pursuit of well-being and longevity has driven a continuous search for innovative functional foods, ingredients, and supplements (Granato et al., 2010; Li et al., 2020). This trend is particularly evident in addressing gastrointestinal ailments, such as allergies and intolerances. Traditional fermented foods constitute a significant portion of functional foods, primarily due to the presence of probiotics. These microorganisms, along with the bioactive molecules secreted during microbial metabolism, provide various health benefits to humans, including prebiotics, enzymes, and other bioactive compounds.

Lactic acid bacteria (LAB) are widely distributed across diverse environments, such as dairy, meat, plants, vegetables, fruits, soil, and water (Ruiz Rodríguez et al., 2019). Their adaptability enables them to colonize numerous ecological niches, including fermented foods. LAB species utilize raw materials such as plants, cereals, fruits, whey, and honey as substrates for fermentation (Garcia-Parrilla et al., 2017). LAB has a long history of use as probiotics, offering a wide range of health benefits to the host (Li et al., 2020). While traditionally associated with milk-based products, such as yogurt and fermented dairy products (Sherwani and Ara Abbas Bukhari, 2016), LAB are also found in various other fermented foods, including vinegar, cocoa, coffee, kombucha, and wine.

Vinegar, for instance, is one of the oldest fermented foods, with a history dating back to 2000 BCE, and has been consumed for its health benefits since ancient times (Giudici, 2019). In this context, Chapter 1 of this thesis aims to explore the presence of LAB in vinegar and identify potential probiotic strains. Furthermore, it investigates the possibility of positioning vinegar as a functional food and a complementary postbiotic supplement in human nutrition.

Given the significant presence of LAB in natural apple vinegar—a factor that enhances its potential as a functional food—exploring their enzymatic production capabilities is advantageous. Although human digestion primarily relies on gastrointestinal enzymes, the gut microbiota—including bacteria and fungi—plays a complementary role in producing digestive enzymes (García-Cano et al., 2020). It could produce digestive-associated enzymes such as lactase, proteases, peptidases, fructanases, amylases, bile salt hydrolases, phytases, and esterases. In this way, Chapter 2 underscores the potential of lactobacilli, as part of resident in the human GIT from its initial colonization (Xanthopoulos et al., 2000; Pereira et al., 2018) to secrete functional enzymes integral to digestion, and examines how leveraging this metabolic complexity can enhance human nutrition.

Over the past two decades, significant progress has been made in the study of fermented foods, largely due to advancements in next-generation sequencing, advanced mass analyzers, and other innovative tools (Xanthopoulos, et al., 2000). Next-generation sequencing (NGS) is particularly valuable for accessing and investigating microbiota within a food matrix. This technique allows for the mapping of LAB and other major microbial groups involved in the fermentation process. Additionally, NGS enables the quantification of minor microbial groups, including environmental contaminants commonly found in traditionally fermented foods, as well as pathogenic bacteria and fungi. High-throughput sequencing has frequently revealed numerous previously undetected, non-dominant microbes (Pereira et al., 2018).

The microbial composition of vinegar, for instance, directly impacts the product's quality and the formation of volatile compounds during fermentation (Gongo et al., 2023). Although LAB are present in lower proportions compared to the dominant acetic acid bacteria, they play a significant role in shaping the vinegar's sensory profile Thierry et al., 2015). In this context, the fermentation process of natural apple vinegar was analyzed over three months, as described in Chapter 3, to assess the incidence of LAB and identify the groups driving the fermentation. Volatile compounds were also analyzed via GC-MS, and substrate consumption and organic acid formation were assessed using HPLC. Additionally, correlation tests were performed to understand which groups coexist or exhibit negative relationships, as well as which groups are associated with specific volatile compounds.

The emergence of Coronavirus Disease 2019 (COVID-19) as a global pandemic has heightened public concerns about diseases caused by viruses. Fermented foods, which contain high loads of viable fungi and bacteria (Pereira et al., 2020), can also serve as potential sources of viral contamination. Fermented foods serve as a rich reservoir for the proliferation of viruses that infect various microorganisms. Notably, studies have documented the presence of bacteriophages and viruses targeting yeasts in a wide range of fermented food products, such as wine, meat, cheese, yogurt, sourdough, sauerkraut, kimchi, soybean-based products, and cocoa (Auad et al., 1997; Barrangou et al., 2002; Foschino et al., 2005; Illeghems et al., 2012; Kiliç et al., 1996; Kleppen et al., 2012a; Pringsulaka et al., 2011; Umene et al., 2009). Generally, bacteriophages are regarded as detrimental, as they can impair the fermentative capacity of lactic acid bacteria (LAB) and yeasts, potentially leading to fermentation failure. An extensive review and analysis were conducted to investigate the presence of bacteriophages and viruses that infect yeasts with fermented foods, as well as potential pathogenic viruses. This investigation, detailed in Chapter 4, aimed to evaluate the viral content in various fermented products and assess their potential impact on food quality and production. Notably, these viruses can have both positive and negative effects, influencing fermentation processes either by enhancing microbial interactions or by disrupting the activity of key fermentative microorganisms.

Chapter 4 presented a study reporting a yeast virus in wine (Rodríguez-Cousiño et al., 2011). Fungal viruses are primarily double-stranded RNA (dsRNA) or positive single-stranded RNA (+ssRNA), though some have linear negative single-stranded RNA (-ssRNA) and circular single-stranded DNA (ssDNA) genomes (Ghabrial et al., 2015). These viruses have also been identified in yeasts (*S. cerevisiae*), where they are referred to as 'yeast viruses' in the context of winemaking (Ramírez et al., 2015; Rodríguez-Cousiño et al., 2011). Unlike bacteriophages, *S. cerevisiae* viruses belong to the *Totiviridae* family (order *Ghabrivirales*) and can infect not only yeasts but also protozoa, filamentous fungi, plants, invertebrates, and vertebrates (Rowley, 2017). Although most mycoviruses appear to be harmless, they can induce phenotypic changes in their hosts, such as pigmentation abnormalities, altered growth rates, variations in sporulation, modified stress tolerance, hypo- or hypervirulence, or even enabling the production of extracellular antifungal toxins (Jagdale and Joshi, 2015). Given their importance in virome studies, yeast viruses and their implications in fermented foods and beverages were discussed in Chapter 5.

The investigation of viruses in fermented foods has traditionally depended on culture-based methods, primarily targeting individual bacteriophages associated with fermentation defects and human pathogenic viruses (Park et al., 2011). However, advancements in molecular techniques, coupled with the development of next-generation sequencing (NGS), have enabled the characterization and emergence of "viromes" (Ledormand et al., 2020; Tamang et al., 2020). Shotgun metagenomics, an approach that does not rely on specific ribosomal markers, has been successfully employed to characterize viral communities in diverse environments, including freshwater, soil, oceans, and the mammalian gut, with limited application to fermented food products (Dugat-Bony et al., 2020; Hayes et al., 2017; Park et al., 2011). In Chapter 6 the virome analysis was conducted on the natural vinegar fermentation matrix to assess the viral content, with a focus on the presence of bacteriophages and phage-bacteria interactions, aiming to understand how this community may influence the beverage. To achieve this, KEGG analyses were performed to explore the roles of bacterial and fungal microbial communities and to evaluate how phages shape the fermentation process.

In summary, this study aimed to enhance the understanding of the role of microbiome in fermented food, focusing on apple vinegar research by investigating the total microbial composition present as well as their correlation applications. It further explored how these insights could contribute to advancing food technologies and improving food safety.

CHAPTER ONE – EXPLORING DIVERSITY AND FUNCTIONAL TRAITS OF LACTIC ACID BACTERIA IN TRADITIONAL VINEGAR FERMENTATION: A REVIEW

Manuscript published in *International Journal of Food Microbiology* journal. DOI: https://doi.org/10.1016/j.ijfoodmicro.2023.110550.

Abstract

Vinegar has been used for centuries as a food preservative, flavor enhancer, and medicinal agent. While commonly known for its sour taste and acidic properties due to acetic acid bacteria metabolism, vinegar harbors a diverse community of lactic acid bacteria (LAB). The main genera found during natural fermentation include *Lactobacillus*, *Lacticaseibacillus*, *Lentilactobacillus*, *Limosilactbacillus*, *Leuconostoc*, and *Pedicoccus*. Many of the reported LAB strains fulfill the probiotic criteria set by the World Health Organization (WHO). However, it is crucial to acknowledge that LAB viability undergoes a significant reduction during vinegar fermentation. While containing LAB, none of the analyzed vinegar met the minimum viable amount required for probiotic labeling. To fully unlock the potential of vinegar as a probiotic, investigations should be focused on enhancing LAB viability during vinegar fermentation, identifying strains with probiotic properties, and establishing appropriate dosage and consumption guidelines to ensure functional benefits. Currently, vinegar exhibits substantial potential as a postbiotic product, attributed to the high incidence and growth of LAB in the initial stages of the fermentation process. This review aims to identify critical gaps and address the essential requirements for establishing vinegar as a viable probiotic product. It comprehensively examines various relevant aspects, including vinegar processing, total and LAB diversity, LAB metabolism, the potential health benefits linked to vinegar consumption, and the identification of potential probiotic strains.

Keywords: Lactic acid bacteria; *Lactobacillus;* Acetic acid; Natural fermentation

Introduction

The global pursuit of well-being and longevity has spurred a constant search for novel functional foods, ingredients, and supplements (Granato et al., 2010; M. Li et al., 2020). Lactic acid bacteria (LAB) have a rich history of use as probiotics, offering a myriad of health benefits to the host (M. Li et al., 2020). While LAB has traditionally been associated with milk-based products like yogurts and traditional fermented milks (Sherwani and Ara Abbas Bukhari, 2016), the demand for nondairy has constantly been increasing driven by factors such as veganism, high cholesterol content in milk, and lactose intolerance among individuals (Tadesse and Emire, 2020).

Vinegar, an ancient, fermented food with a history dating back to 2000 BCE, stands as one of the earliest culinary creations (Giudici, 2019). It is believed that a Babylonian courtier accidentally discovered vinegar formed from abandoned wine or unattended grape juice (Johnston and Gaas, 2006). The product was mentioned in the Bible for medical purposes and applied as a remedy due to its sedative and curative properties (Plessi, 2003). In recent years, vinegar has become widely utilized as a flavor enhancer in dishes worldwide, as well as for cleaning surfaces and utensils (Hemke et al., 2019).

The advent of culture-independent sequencing methods has significantly enhanced comprehensive investigations into the diverse microbiota responsible for the natural fermentation of vinegar, complementing the insights gained from conventional culture-dependent methods (Fang et al., 2021). LAB is a notable group in vinegar fermentation, being ahead of acetic acid bacteria (AAB) in diversity matter, and are dominant in numerous vinegar types, such as apple (Trček et al., 2016), rice (Haruta et al., 2006; Shi et al., 2013), cereal (Zhang et al., 2020), Shanxi-aged (Wu et al., 2012), Zhejiang rosy (Fang et al., 2021), Zhenjiang aromatic vinegar (Wang et al., 2016), Qishan (Gan et al., 2017), and Tianjin Duliu (Nie et al., 2013). Probiotics are categorized as live microorganisms that, when administered at a sufficient dose, demonstrate health benefits for the host (Hill et al., 2014). While LAB are highly prevalent during vinegar fermentation, the significant reduction in viable cells in the final product falls short of meeting the required dosage for probiotic labeling (typically $\geq 10^6$ to 10^9 CFU/g or CFU/mL at the time of consumption, depending on the strain and regulatory guidelines).

LAB are widely distributed across diverse environments, such as dairy, meat, plants, vegetables, fruits, soil, and water (Ruiz Rodríguez et al., 2019). Thus, the initial stage of vinegar fermentation serves as a gateway for the introduction of LAB species, utilizing plants, cereals, fruits, whey, and honey as raw materials (Garcia-Parrilla et al., 2017). Typically, the total microbial population present on fresh vegetables and fruits ranges from 10^5 to 10^7 colony-forming units per gram (CFU/g), while specific microbial groups may occur at lower levels, approximately 10^2 CFU/g; among them, yeasts are the predominant $–10^6$ CFU/g; LAB, on the other hand, represent a minor part of the microbiota, ranging between 10^2 and 10^4 CFU/g (Ruiz Rodríguez et al., 2019). Even to a lower degree, it can tread constancy and/or dominance in vinegar fermentation. *Limosilactobacillus fermentum* (former *Lactobacillus fermentum*), *Lactiplantibacillus plantarum* (former *Lactobacillus plantarum*), *Lentilactobacillus buchneri* (former *Latobacillus buchneri*), *Lacticaseibacillus casei* (former *Lactobacillus casei*), *Pediococcus acidilactici*, *P. pentosaceus*, and *Weissella confusa* are some species frequently associated with vinegar fermentation (Wu et al., 2012). However, throughout the fermentation process, LAB encounters inhibitory factors that limit their viability in the final product.

This review aims to compile the various methods of vinegar production, analyze the overall microbial diversity with a specific focus on LAB, and identify potential probiotic strains found in vinegar. By doing so, it aims to explore the possibility of vinegar as a complementary postbiotic supplement to human nutrition.

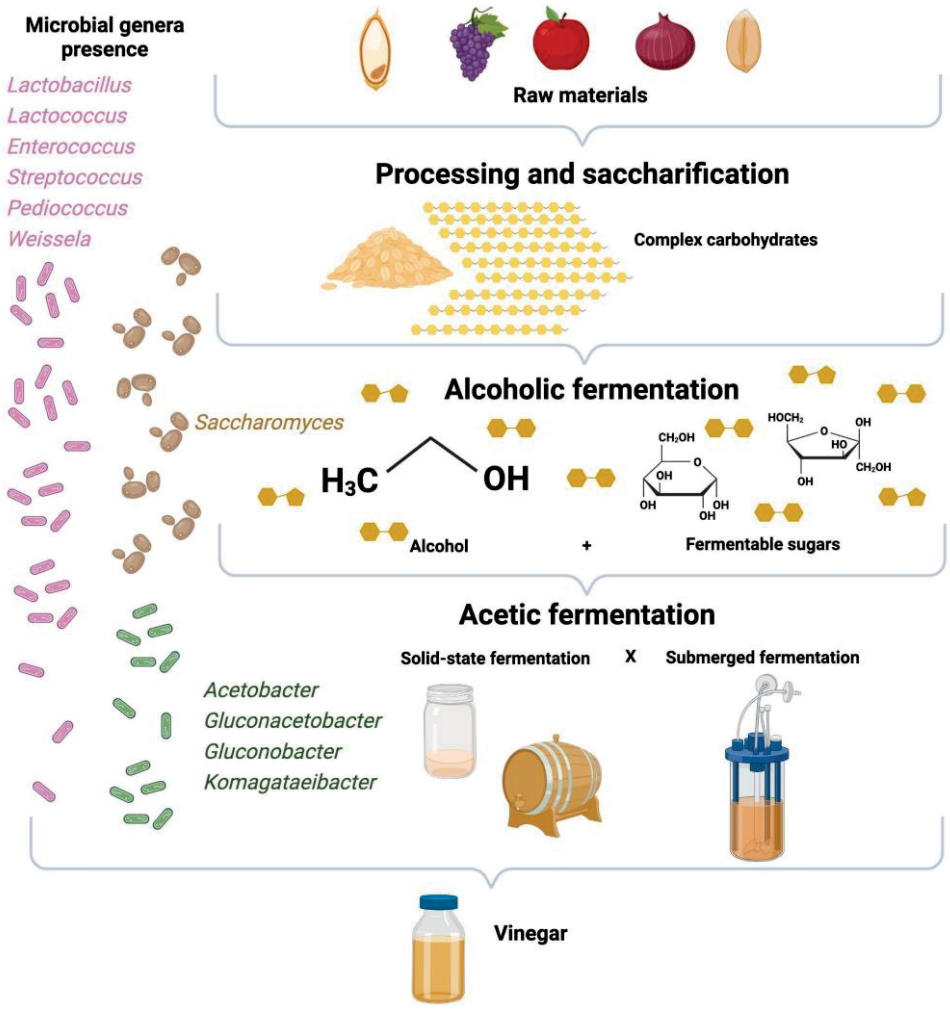


Figure 1. Schematic workflow for processing strategies and microbial main groups dynamics during vinegar production.

Vinegar production methods

A diverse range of raw materials is utilized in vinegar production, encompassing grains (such as rice, malt, and sorghum) (Giudici, 2019; Kandylis et al., 2021), fruits (such as apple, grape, and coconut), vegetables (including onion), animal products (such as honey and whey) (Li et al., 2015), and other sources like sugarcane and roots (Kandylis et al., 2021; Kim et al., 2021). The type of operation is either solid-state fermentation (SSF) or submerged fermentation (SmF) (Figure 1). SSF is a technique that applies low water content – between 30 and 85% (Machado de Castro et al., 2018) – and usually used in traditional open fermentation (Chen et al., 2019). On the other hand, SmF is usually applied in industrial production with higher productivity and yields, and lower process time when compared to SSF (Li et al., 2022).

The fermentation process can occur through spontaneous fermentation, backslopping processes, or the addition of starter cultures. In the case of spontaneous fermentation, external conditions can be adjusted and regulated to facilitate the optimal growth and development of specific microorganisms (Chochevska et al., 2021; Lu et al., 2018). Studies show a high level of microbial diversity in this type of fermentation, resulting, therefore, in a complex combination of metabolites that provide great organoleptic characteristics and functional properties for the final product (Luzón-Quintana et al., 2021). However, a spontaneous process can present the drawback of a higher risk of contamination and long fermentation periods. Backslopping techniques are the precursor of starter cultures, utilizing part of a portion of a previous batch to inoculate a new fermentation process. This practice can expedite fermentation, enabling faster progress and development (Giudici, 2019; Kim et al., 2021). Starter cultures are a set of predefined and well-selected microorganisms that are inoculated at the beginning of different food-related fermentation processes (Durso and Hutkins, 2003). This strategy allows better control and reproducibility of the process, avoids external contamination, and provides similar characteristics for the final product between batches (Kim et al., 2021; Vinicius De Melo Pereira et al., 2019). Nevertheless, the use of starter cultures decreases natural microbial diversity, potentially resulting in a final product that exhibits reduced flavor compared to vinegar produced through open fermentation methods (Cosmulescu et al., 2022).

The vinegar fermentation process encompasses diverse microorganisms, leading to a complex dynamic throughout the fermentation period. This dynamic, which has been extensively studied, involves fluctuations in microbial diversity over time. The employment of specific strains or genera of microorganisms as starter cultures presents a promising strategy to finely tune the dynamics of the fermentation process and achieve vinegar with distinct characteristics and flavors. By introducing carefully selected microbial cultures, the desired metabolic pathways can be enhanced or modified, leading to the production of vinegar with targeted sensory profiles and improved quality. This approach allows for greater control over the fermentation process, enabling producers to tailor vinegar production to meet consumer preferences and market demands (Chai et al., 2020a).

The vinegar production process usually occurs in three steps: saccharification, alcoholic fermentation (AF), and acetic acid fermentation (AAF). Before any

fermentation process, it is common practice to mill the raw material to facilitate the release of carbohydrates and sugars, making them more accessible for microbial action. By breaking down the physical structure of the raw material, milling increases the surface area and exposes a larger portion of the substrate to enzymatic and microbial activities, promoting efficient utilization and conversion of the carbohydrates into desired fermentation products. This step plays a crucial role in optimizing the overall efficiency and effectiveness of the fermentation process, ensuring the maximum utilization of the raw material's nutritional components by the microorganisms involved (Li et al., 2015). Fruits and vegetables usually have their juice extracted already available for fermentation (Ho et al., 2017), while starchy grains require an additional saccharification step. Vegetables and fruits are advantageous matrices for LAB growth as they are composed of simple carbohydrates (fructose and glucose), facilitating the first degradation steps. Grains and cereals, on the other hand, which have more complex carbohydrates to degrade (starch, cellulose, and hemicellulose), depend on the production of specific enzymes for polysaccharide degradation to monosaccharides (J. Wang et al., 2021). This step involves the hydrolysis of these complex carbohydrates into fermentable sugars through the combined enzymatic action, predominantly from amylases (Li et al., 2015; Taweekasemsombut et al., 2021). Saccharification can be achieved by adding commercial enzymes or by utilizing fungi that naturally produce extracellular enzymes, such as those from the *Aspergillus* and *Rhizopus* genera. When fungi are involved in saccharification, other metabolites that provide flavor and color can also be released, influencing the vinegar's final characteristics (Li et al., 2015). Some LAB, with a notable emphasis on specific members of the *Lactobacillus* genera such as *Limosilactobacillus amylovorus* (formerly *Lactobacillus amylovorus*), *Lactiplantibacillus plantarum* (formerly *Lactobacillus plantarum*), *Limosilactobacillus manihotivorans* (formerly *Lactobacillus manihotivorans*), and *Limosilactobacillus fermentum* (formerly *Lactobacillus fermentum*), possess significant amylolytic activity. This enzymatic capability enables them to fulfill a similar role as fungi, suggesting a promising alternative to mold enzymes in various applications (J. Wang et al., 2021).

The AF is the metabolic process in which fermentable sugars are anaerobically converted into ethanol by yeasts. During the fermentation process, a variety of yeasts are involved, including *Saccharomyces*, *Zygosaccharomyces*, *Kluyveromyces*, and *Pichia*. Non-*Saccharomyces* yeasts are typically more abundant in the early and middle stages of

fermentation. However, as the fermentation progresses, *Saccharomyces* (mainly *S. cerevisiae*), become dominant due to its alcohol tolerance (Li et al., 2015).

The pH of the fermentation typically ranges between 3 and 5, while the sugar content is meticulously maintained at approximately 20 %. This control is crucial to prevent the sugar content from exceeding the designated threshold, thereby avoiding excessive osmotic pressure and subsequent microbial inhibition (Luzón-Quintana et al., 2021). Raw materials with higher sugar concentrations (e.g honey) should be diluted to prevent prolonged fermentation process (Perumpuli and Dilrukshi, 2022).

AAF occurs through the aerobic oxidation of ethanol to acetic acid. This reaction takes longer than AF and is usually conducted at an acidic pH. The process can occur in SSF, with the microorganisms being cultivated in the raw material surface (Li et al., 2015) or in a SmF, with the filtrate derived from AAF being fermented (Gullo et al., 2014). Most of the compounds that guarantee vinegar's unique flavor and aroma (organic acids and volatiles) are produced in the AAF. The main microorganisms involved in this process are AAB from the genera *Acetobacter*, *Gluconobacter, Gluconoacetobacter* and *Komagataeibacter*, each one having its specific characteristics. *Acetobacter* is the most common AAB found (Chai et al., 2020b; Chen et al., 2017) and is present mainly at the beginning of the process, while *Komagataeibacter* (a new genus that includes several AAB, mostly transferred from *Gluconoacetobacter*) is resistant to high concentrations of acetic acid and is therefore predominant at late process stages (Li et al., 2015). *Gluconobacter*, in turn, can oxidize a more significant number of substrates, including glucose which is converted to gluconic acid (a compound with interesting health properties), and is resistant to high alcohol concentrations, making it suitable for both AF and early-stage AAF processes (Li et al., 2015; Luzón-Quintana et al., 2021). Apart from acetic acid, AAF can yield other important metabolites. Among them, glycerol stands out as it can be further utilized for the generation of flavor-active esters. Additionally, smaller quantities of volatiles such as ethyl acetate, acetaldehyde, methanol, and furfural can also be produced during the fermentation process. These compounds contribute to the overall aroma and sensory profile of the final vinegar product (Spinosa et al., 2015).

LAB plays a crucial role in vinegar production by producing lactic acid and other active molecules, which contribute to the preservation and development of distinctive flavors in vinegar (Chai et al., 2020b; Li et al., 2022; Wu et al., 2021). Among LAB, the most found genera include *Lactococcus lactis* (formerly *Lactobacillus lactis*),

Lentilactobacillus acetotolerans (formerly *Lactobacillus acetotolerans*), *Lacticaseibacillus casei* (formerly *Lactobacillus casei*), and *Lactiplantibacillus plantarum* (formerly *Lactobacillus plantarum*), as well as *Pediococcus* and *Leuconostoc*, although to a lesser extent (Li et al., 2015). These LAB species are typically present during the early and middle stages of fermentation, where they interact symbiotically with yeasts (Li et al., 2015). Their metabolic activities contribute to the acidification process, flavor development, and overall quality of vinegar.

Post-fermentation processes typically encompass the ripening stages of the resulting product. During this period, the acetic acid content becomes concentrated, while additional aromatic active compounds such as furans and esters are generated, contributing to the enhanced complexity of the product (Spinosa et al., 2015). The technological steps, raw materials, and physicochemical conditions employed in vinegar fermentation directly impact on the microbial diversity and dynamics throughout the production process. Consequently, these factors significantly influence the metabolites produced and the final vinegar product's overall quality and characteristics (Li et al., 2015).

Health benefits of vinegar consumption

While vinegar is primarily known for its usage as a food condiment and preservation purposes, it has also found extensive application as a traditional medicine in various countries. Vinegar consumption has been demonstrated to induce a variety of *in vivo* and i*n vitro* activities, extending beyond mere ingestion. Its adaptability extends to topical application on burns and infectious tissues, where it harnesses antibacterial properties and fosters tissue repair. This reparative capability is ascribed to the extracellular structure synthesized by *Acetobacter* species (Budak et al., 2014). These endeavors seek to enhance our understanding of the underlying mechanisms that drive the therapeutic effects of this unique food condiment.

Vinegar oral consumption demonstrated numerous advantages in animal models. Anti-diabetic effect on rat bloodstream sugar was reported within 24 h of starch ingestion supplemented with a 2% acetic acid solution when compared with a normal diet (Ebihara and Nakajima, 1988). This effect may be a consequence of acetic acid blocking the complete digestion of complex carbohydrates, accelerating gastric emptying or increasing tissue glucose absorption, resulting in reduced blood glucose levels (Budak et al., 2014).

Red wine vinegar demonstrated a hypotensive effect by inhibiting the renin-angiotensin system in rats (Honsho et al., 2005). A reduction in pressor response induced by angiotensin I, decreased from 572 to 457 mmHg, was observed in 3 mL of vinegar consumption per kg after 60 min. Also, vinegar may assist in the protection of organs against injuries related to oxidative stress (Perumpuli and Dilrukshi, 2022). Daily ingestion of vinegar containing acetate 5% as the principal bioactive molecule resulted in suppression of kidney stone formation (phosphate, urate and crystals of calcium oxalate) in rats after 4 weeks of oral administration. It has been suggested that an excretion of urinary calcium from the gastrointestinal tract (Zhu et al., 2019).

Organic acids and bioactive components (e.g., acetic acid, gallic acid, catechin, epicatechin, chlorogenic acid, caffeic acid, p-coumaric acid, and ferulic acid) (Budak et al., 2014), on the other hand, can play a crucial role in various physiological processes. Firstly, they have the potential to lower the gastrointestinal pH, thereby enhancing the body's ability to absorb minerals (Safari et al., 2017). Additionally, these acids can inhibit digestive amylases responsible for breaking down complex sugars into glucose, consequently reducing blood glucose levels (Perumpuli and Dilrukshi, 2022; Samad et al., 2016). Furthermore, organic acid's bacteriostatic and bactericidal properties hinder the proliferation of harmful bacteria, ensuring the maintenance of a healthy microbial balance (Bakir et al., 2017).

Acetate can help prevent kidney stone formation through the excretion of calcium (Zhu et al., 2019), reducing glucose blood levels by helping its conversion to glycogen (Chen et al., 2016), and even restrain cancer cells proliferation (Samad et al., 2016). Vinegar can also promote anti-inflammatory and anticancer activities through several acting mechanisms and metabolites (Perumpuli and Dilrukshi, 2022).

As summarized, vinegar's consumption benefits come mainly from the metabolites present in the product. However, the presence of LAB as probiotics can also bring new advantages. Along with helping to maintain the intestinal flora active and in balance and hindering the multiplication of pathogenic bacteria, probiotics may present antioxidant properties (Wang et al., 2017), decrease cholesterol levels (Ishimwe et al., 2015), and increase insulin sensitivity. Sui et al., (2021) comprehensively evaluated several properties of *Lactobacillus* strains isolated from tangerine vinegar. The results revealed that all the strains exhibited remarkable antioxidant and antibacterial properties and the ability to remove cholesterol. Is important to mention that these results have not

been fully demonstrated in humans. The studies conducted so far are preliminary, and this research does not recommend the consumption of vinegar as a treatment or preventive measure for any diseases.

Total bacterial composition

Vinegars harbor rich microbiota in their matrices, including bacteria, filamentous fungi and yeasts. Plenty of vinegars had their microbiota explored in detail, such as apples (Trček et al., 2016), rice (Haruta et al., 2006; Shi et al., 2013), cereal (Zhang et al., 2020), Shanxi-aged (Wu et al., 2012), Zhejiang rosy (Fang et al., 2021), Zhenjiang aromatic (Wang et al., 2016), Qishan (Gan et al., 2017), and Tianjin Duliu (Nie et al., 2013).

Vinegar has attracted substantial research interest, particularly in China, where it has emerged as a leading focus of study. The survey conducted on the bacterial microbiota of these products unveiled an astonishing diversity, revealing the presence of over 80 distinct genera and 50 species, as shown in Tables 1, 2, 3 and 4. The vinegar fermentation process occurs statically in urns (Chai et al., 2020a) for approximately one month, being early stage classified from day 0 until day 4 (Zhu et al., 2018), the middle stage around day 5 to 15th days (Xu et al., 2011; Zhu et al., 2018), and later stage days 22 to 26 (Zhu et al., 2018).

The deep investigation of vinegar's bacterial profile during the process was possible by performing sequencing methods. Following the discoveries timeline, independent cultivation methods, such as PCR, qPCR, and PCR-DGGE, unveiled many different groups (Haruta et al., 2006; Shi et al., 2013.; Xu et al., 2011; Zhu et al., 2018), and posteriorly, the ascension of next-generation sequencing platforms, such as Pyrosequencing, Illumina MiSeq and Illumina HiSeq (Xia et al., 2016), raised plenty of other microbial groups. These platforms are capable of producing high numbers of DNA sequences in the library preparation, allowing an in-depth description of the microbial constituents of different fermented foods (de Melo Pereira et al., 2020b) such as sauerkraut and kimchi (Srinivas et al., 2022), fermented milk (Maske et al., 2021b), and coffee (da Silva Vale et al., 2023).

AAB is the bacterial group primarily associated with vinegar fermentation, and its role in the process has been extensively established (Perumpuli and Dilrukshi, 2022). In the middle to final stages of fermentation, the dominant microbial groups belong to the *Acetobacter* genus, although contributions from *Gluconacetobacter*, *Gluconobacter*, and *Komagataeibacter* have also been observed. These microorganisms play a pivotal role in converting ethanol to acetic acid.

Table 1 shows the diversity of these groups for each type of vinegar. *A. pasteurianus* is prevalent in almost all types of vinegar, while *A. aceti* and *A. syzygii* are present to a lesser extent. Shanxi-aged vinegar presents the greatest diversity of this genus, including *A. senegalensis*, *A. indonesiensis*, *A. malorum*, *A. orientalis* and *A. pomorum*. Nevertheless, AAB, despite being dominant and playing a primary role in acetic acid fermentation, are present throughout all stages of the fermentation process.

Table 1. Vinegar AAB diversity.

AAB genus/species	Vinegar type	Fermentation stage	Sequencing method	Dominance (Yes/No/Constant)	References
Acetobacter	Zhenjiang aromatic vinegar	Later stage of acetic acid fermentation (days 10–18)	Illumina MiSeq/ qPCR	Yes	(Wang et al., 2016)
	Slovene apple vinegar	Acid acetic fermentation	DHPLC/ Illumina MiSeq	Yes	(Trček et al., 2016)
	Zhejiang rosy vinegar	Acetic acid fermentation	llumina MiSeq	Yes	(Fang et al., 2021)
	Apple vinegar	Acetic acid fermentation	Illumina MiSeq	Yes	(Song et al., 2019)
	Shanxi aged vinegar	Later stage (days 22–26)	PCR-DGGE/ Pyrosequencing	Yes	(Zhu et al., 2018)
	Vinegar Pei	Acetic acid fermentation	Pyrosequencing	Yes	(Wang et al., 2015)
	Tianjin duliu vinegar	Acetic acid fermentation	PCR-DGGE/ Illumina	Yes	(Nie et al., 2013)
	Zhenjiang aromatic vinegar	Acid acetic fermentation	PCR-DGGE	NM	(Xu et al., 2011)

	Qishan vinegar	Acetic acid fermentation	qPCR/ Illumina HiSeq	Yes	(Gan et al., 2017)
	Tianjin Duliu aged vinegar	Acetic acid fermentation	PCR-DGGE/ 454-Pyrosequencing	Yes	(Peng et al., 2015)
	Zhenjiang aromatic vinegar	All stages	Illumina MiSeq/ qPCR	No	(Wang et al., 2016)
Acetobacter pasteurianus	Rice vinegar	Early stage	PCR-DGGE	Yes	(Haruta et al., 2006)
	Meigui Rice Vinegar	Final stage	PCR-DGGE	Yes/ Constant	(Shi et al., 2013)
	Rice Vinegar Meiguichu	Acetic acid fermentation	Illumina HiSeq	NM	(Xia et al., 2016)
	Apple-based kefir vinegar	All stages	MALDI-TOF MS	Constant	(Viana et al., 2017)
	Chinese cereal vinegar	Alcohol and acetic acid fermentation	Illumina MiSeq	Yes	(Zhang et al., 2020)
	Tianjin duliu vinegar	Acid acetic fermentation	PCR-DGGE/ Illumina	Yes	(Nie et al., 2013)
	Shanxi aged vinegar	Acid acetic fermentation	Inter-delta PCR/ PCR-RFLP/ ERIC-PCR/ 16S rRNA and 26S rRNA partial gene sequencing	Yes	(Wu et al., 2012)

	Japanese Rice Vinegar	All stages	PCR	Yes	(Nanda et al., 2001)
	Zhenjiang aromatic vinegar	Acid acetic fermentation	PCR-DGGE	NM	(Xu et al., 2011)
Acetobacter aceti	Meigui Rice Vinegar	Final stage	PCR-DGGE	No	(Shi et al., 2013)
Acetobacter syzygii	Apple-based kefir vinegar	All stages	MALDI-TOF MS	Constant	(Viana et al., 2017)
Acetobacter senegalensis	Shanxi aged vinegar	Initial point of alcoholic	Inter-delta PCR/ PCR-RFLP/ ERIC-PCR/ 16S rRNA and 26S rRNA partial gene sequencing	No	(Wu et al., 2012)
Acetobacter indonesiensis	Shanxi aged vinegar	Initial point of alcoholic fermentation	Inter-delta PCR/ PCR-RFLP/ ERIC-PCR/ 16S rRNA and 26S rRNA partial gene sequencing	No	(Wu et al., 2012)
Acetobacter malorum	Shanxi aged vinegar	Initial point of alcoholic fermentation	Inter-delta PCR/ PCR-RFLP/ ERIC-PCR/ 16S rRNA and 26S rRNA partial gene sequencing	No	(Wu et al., 2012)
Acetobacter orientalis	Shanxi aged vinegar	Initial point of alcoholic fermentation	Inter-delta PCR/ PCR-RFLP/ ERIC-PCR/ 16S rRNA and 26S rRNA partial gene sequencing	No	(Wu et al., 2012)
Acetobacter pomorum	Zhenjiang aromatic vinegar	Acid acetic fermentation	PCR-DGGE	NM	(Xu et al., 2011)

Gluconacetobacter	Tianjin duliu vinegar	Acetic acid fermentation	PCR-DGGE/ Illumina	Yes	(Nie et al., 2013)
	Zhenjiang aromatic vinegar	Acid acetic fermentation	PCR-DGGE	NM	(Xu et al., 2011)
	Tianjin Duliu aged vinegar	All stages	PCR-DGGE/ 454-Pyrosequencing	No	(Peng et al., 2015)
	Zhenjiang aromatic vinegar	Later stage of acetic acid fermentation (days 10–18)	Illumina MiSeq/ qPCR	No	(Wang et al., 2016)
Gluconacetobacter intermedius	Zhenjiang aromatic vinegar	Middle stage (15th day)	PCR-DGGE	NM	(Xu et al., 2011)
Gluconobacter	Slovene apple vinegar	Acid acetic fermentation	DHPLC/ Illumina MiSeq	No	(Trček et al., 2016)
Gluconobacter oxydans	Shanxi aged vinegar	Initial point of alcoholic fermentation	Inter-delta PCR/ PCR-RFLP/ ERIC-PCR/ 16S rRNA and 26S rRNA partial gene sequencing	No	(Wu et al., 2012)
	Meigui Rice Vinegar	Final stage	PCR-DGGE	No	(Shi et al., 2013)
Komagataeibacter	Slovene apple vinegar	Acid acetic fermentation	DHPLC/ Illumina MiSeq	Yes	(Trček et al., 2016)

	Shanxi aged vinegar	Middle and later stage	PCR-DGGE/ Pyrosequencing	No	(Zhu et al., 2018) .
	Chinese cereal vinegar	Alcohol and acetic acid fermentation	Illumina MiSeq	Yes	(Zhang et al., 2020)
	Shanxi aged vinegar	Later stage, days 22–26	PCR-DGGE/ Pyrosequencing	Yes	(Zhu et al., 2018)
	Zhejiang rosy vinegar	Early alcoholic fermentation	llumina MiSeq	No	(Fang et al., 2021)
Komagataeibacter oboediens (formerly *Gluconacetobacter oboediens)*	Slovene apple vinegar	All stages	DHPLC/ Illumina MiSeq	Yes	(Trček et al., 2016)

NM: Not mentioned; DHPLC: Denaturing high performance liquid chromatography; MALDI-TOF MS: Matrix-assisted laser desorption ionization–time-of-flight mass spectrometry; PCR: Polymerase chain reaction; DGGE: Denaturing Gradient Gel Electrophoresis; RFLP: Restriction Fragment Length Polymorphism; ERIC-PCR: Enterobacterial Repetitive Intergenic Consensus Polymerase Chain Reaction; qPCR: quantitative PCR

Regardless of principal groups, natural fermentations are prone to many microorganisms in lesser loads. They may have different inoculation origins, such as raw materials/food, the environment surrounding the fermentation process, the utensils used, and even human contact (Voidarou et al., 2021). The microbial diversity present in vinegar production plays a pivotal role in shaping its distinctive organoleptic characteristics while upholding stringent safety standards. However, potential routes for environmental contaminants, as well as opportunistic or foodborne pathogenic microorganisms, should be considered (Maske et al., 2021a). LAB and AAB can eventually avoid or reduce the existence of these strains through the production of acids and antimicrobial substances during fermentation (Djadouni and Kihal, 2012; Kandylis et al., 2021). However, some persist until the final stage. *Escherichia* and *Shigella*, known as opportunistic bacteria, are constant in all stages of apple vinegar (Song et al., 2019). *Pseudomonas* genera, housing pathogenic species within the group, are present in all stages of Zhenjiang aromatic vinegar (Wang et al., 2016), Tianjin Duliu aged vinegar (Peng et al., 2015), Daqu starter (Tang et al., 2019), and even constant in Zhejiang rosy vinegar fermentation (Fang et al., 2021).

Environmental bacterial groups in vinegar diversity are compiled in Table 2. They are distributed in nature and can have multiple sources, such as water, soil, plant, food, air, animals, and even utensils and recipients. The *Bacillus* genus emerges as the prevailing environmental microorganism found in vinegar, capable of existing in all stages of fermentation, either as the dominant species or in varying proportions. The species found in this genus were *B. licheniformis* and *B. amyloliquefaciens* in Daqu starter (Li et al., 2019). Despite few pathogenic members, *Bacillus* is found in the air, water, soil or dust and is adaptable to various environments (Cote et al., 2014). This genus was attributed as core microbiota and dominant in the production of flavors in Zhenjiang aromatic vinegar (Wang et al., 2016). Also, it was dominant in Qishan vinegar during the AAF process (Gan et al., 2017). The likely source of inoculation can be traced back to the raw materials used in the process.

The second most frequent environmental groups in vinegar are *Acinetobacter* in all stages of fermentation and *Streptomyces* in the early stages. *Acinetobacter* is a food contaminant (Voidarou et al., 2021) and has some resistance to acidic food matrices. It

presented positive correlations with acetic acid in the fermentation of Chinese cereal vinegar (Wu et al., 2021) and traditional Shanxi-aged vinegar (Nie et al., 2017). As *Acinetobacter* species do not secrete acetic acid, this positive correlation suggests a tolerance for acetic acid presence (Nie et al., 2017). This characteristic contributes to the persistence of this group as a contaminant in natural vinegar fermentation. In contrast, *Streptomyces*, often considered an environmental contaminant microorganism, can serve as a pivotal fermenter during the initial stage of vinegar production, owing to its inherent potential for starch saccharification (Nie et al., 2013). They secret alpha-galactosidase enzymes, which could hydrolyze raffinose family oligosaccharides (mainly raffinose and stachyose) (Nie et al., 2013). This member probably originated from the alcohol mash (Zhu et al., 2018) and was dominant in Duliu-daqu, the starter for Tianjin Duliu mature vinegar (Nie et al., 2013). The main species were *S. rangoonensis*, *S. cacaoi*, *S. gibsonii*, *S. radiopugnans*, and *S. albus* (Nie et al., 2013). *Streptomyces* was observed during the first four days of vinegar Pei production. It decreased as the acid acetic fermentation proceeded (Zhu et al., 2018), suggesting its role in the degradation of raw material in the first moment of fermentation.

Additional environmental species include *Rhizobium*, *Chryseobacterium*, *Pantoea*, *Methylobacterium*, *Halomonas*, and *Xanthomonas*. In addition, *Streptomyces*, *Rhizobium*, and *Xanthomonas* were also observed in vinegar Pei in the first four hours (Zhu et al., 2018). This group seems to contribute significantly to flavor formation. It presented, along with *Pantoea,* a positive correlation with 2-octanone substance (Zhu et al., 2018), as well as relevant esters production, including ethyl acetate, hexanoic acid ethyl ester, propanoic acid 2-hydroxy-ethyl ester, ethanol, and 3-methyl-1-butanol. These findings suggest its potential role in esterification processes (Zhu et al., 2018). *Pantoea* is part of the main representative bacteria in Daqu, the starter used in traditional Chinese vinegar production (Li et al., 2019). *Methylobacterium* can influence vinegar flavor, as it is used in the food industry to produce 2,5- dimethyl-4-hydroxy-2H-furan-3-one, a compound known for imparting a delightful strawberry flavor (Wang et al., 2015). In any way, contaminant groups are susceptible to disappearing during fermentation. *Chryseobacterium* disappears by the beginning of the ethanol oxidation phase. LAB's presence is highly influential in inhibiting numerous strains of *Rhizobium, Pantoea,* and *Methylobacterium* (Nie et al., 2017).

Table 2. Vinegar environmental bacteria diversity.

Environmental genus/species	Vinegar type	Fermentation stage	Sequencing method	Dominance (Yes/No/Constant)	References
Bacillus	Apple vinegar	NM	Illumina MiSeq	No	(Song et al., 2019)
	Zhejiang rosy vinegar	All stages	llumina MiSeq	Constant	(Fang et al., 2021)
	Qishan vinegar	Acetic acid fermentation	qPCR/ Illumina HiSeq	Yes	(Gan et al., 2017)
	Tianjin Duliu aged vinegar	All stages	PCR–DGGE and 454-pyrosequencing	No	(Peng et al., 2015)
	Shanxi aged vinegar	All stages	PCR-DGGE/ Pyrosequencing	Yes	(Zhu et al., 2018)
	Daqu starter	All stages	Illumina HiSeq	No	(Tang et al., 2019)
	Zhenjiang aromatic vinegar	Later stage of acetic acid fermentation (days 10–18)	Illumina MiSeq/ qPCR	No	(Wang et al., 2016)
	Daqu starter	NM	PCR-DGGE	Yes	(Li et al., 2019)
Bacillus licheniformis	Daqu starter	NM	PCR-DGGE	No	(Li et al., 2019)
Bacillus amyloliquefaciens	Daqu starter	NM	PCR-DGGE	No	(Li et al., 2019)
Acinetobacter	Apple vinegar	NM	Illumina MiSeq	No	(Song et al., 2019)
	Zhejiang rosy vinegar	All stages	llumina MiSeq	Constant	(Fang et al., 2021)
	Vinegar Pei	NM	Pyrosequencing	No	(Wang et al., 2015)

	Tianjin duliu vinegar	Acid acetic fermentaton	PCR-DGGE/ Illumina	No	(Nie et al., 2013)
	Qishan vinegar	Acetic acid fermentation	qPCR/ Illumina HiSeq	No	(Gan et al., 2017)
	Tianjin Duliu aged vinegar	Acetic acid fermentation	PCR-DGGE/ 454-Pyrosequencing	No	(Peng et al., 2015)
	Tianjin Duliu aged vinegar	Middle stage	PCR-DGGE/ 454-Pyrosequencing	No	(Tang et al., 2019)
	Daqu starter	All stages	Illumina HiSeq	No	(Tang et al., 2019)
Streptomyces	Tianjin duliu vinegar	Early stages	PCR-DGGE/ Illumina	Yes	(Nie et al., 2013)
	Qishan vinegar	Acetic acid fermentation	qPCR/ Illumina HiSeq	Yes	(Gan et al., 2017)
	Shanxi aged vinegar	Early stage (days 0-4)	PCR-DGGE/ Pyrosequencing	No	(Zhu et al., 2018)
Streptomyces rangoonensis	Tianjin duliu vinegar	Early stages	PCR-DGGE/ Illumina	No	(Nie et al., 2013)
Streptomyces cacaoi	Tianjin duliu vinegar	Early stages	PCR-DGGE/ Illumina	No	(Nie et al., 2013)
Streptomyces gibsonii	Tianjin duliu vinegar	Early stages	PCR-DGGE/ Illumina	No	(Nie et al., 2013)
Streptomyces radiopugnans	Tianjin duliu vinegar	Early stages	PCR-DGGE/ Illumina	No	(Nie et al., 2013)
Streptomyces albus	Tianjin duliu vinegar	Early stages	PCR-DGGE/ Illumina	No	(Nie et al., 2013)
Methylobacterium	Tianjin Duliu aged vinegar	Middle stage	PCR-DGGE/ 454-Pyrosequencing	No	(Peng et al., 2015)
	Zhenjiang aromatic vinegar	All stages	Illumina MiSeq/ qPCR	No	(Wang et al., 2016)
	Shanxi aged vinegar	Early stage (days 0-4)	PCR-DGGE/ Pyrosequencing	No	(Zhu et al., 2018)

Pantoea	Daqu starter	NM	PCR-DGGE	Yes	(Li et al., 2019)
	Shanxi aged vinegar	Early stage (days 0-4)	PCR-DGGE/ Pyrosequencing	Yes	(Zhu et al., 2018)
Rhizobium	Shanxi aged vinegar	Early stage (days 0-4)	PCR-DGGE/ Pyrosequencing	Yes	(Zhu et al., 2018)
	Zhenjiang aromatic vinegar	All stages	Illumina MiSeq/ qPCR	No	(Wang et al., 2016)
Chryseobacterium	Apple vinegar	NM	Illumina MiSeq	No	(Song et al., 2019)
	Vinegar Pei	NM	Pyrosequencing	No	(Wang et al., 2015)
Halomonas	Tianjin Duliu aged vinegar	Middle stage	PCR-DGGE/ 454-Pyrosequencing	No	(Peng et al., 2015)
	Zhejiang rosy vinegar	All stages	llumina MiSeq	Constant	(Fang et al., 2021)
Pelomonas	Tianjin Duliu aged vinegar	Acetic acid fermentation	PCR-DGGE/ 454-Pyrosequencing	No	(Peng et al., 2015)
Xanthomonas	Zhenjiang aromatic vinegar	All stages	Illumina MiSeq/ qPCR	No	(Wang et al., 2016)
	Shanxi aged vinegar	Early stage (days 0-4)	PCR-DGGE/ Pyrosequencing	No	(Zhu et al., 2018)
Limnobacter	Apple vinegar	NM	Illumina MiSeq	No	(Song et al., 2019)
Myroides	Apple vinegar	NM	Illumina MiSeq	No	(Song et al., 2019)
Opitutus	Apple vinegar	NM	Illumina MiSeq	No	(Song et al., 2019)
Candidatus	Apple vinegar	NM	Illumina MiSeq	No	(Song et al., 2019)
Cetobacterium	Apple vinegar	NM	Illumina MiSeq	No	(Song et al., 2019)
Variovorax	Apple vinegar	NM	Illumina MiSeq	No	(Song et al., 2019)

Flavobacterium	Zhenjiang aromatic vinegar	Acid acetic fermentation	PCR-DGGE	NM	(Xu et al., 2011)
Cupriavidus	Zhejiang rosy vinegar	Early alcoholic fermentation	llumina MiSeq	No	(Fang et al., 2021)
Brachybacterium	Chinese cereal vinegar	Alcohol and acetic acid fermentation	Illumina MiSeq	No	(Zhang et al., 2020)
Sinorhizobium	Zhenjiang aromatic vinegar	Acid acetic fermentation	PCR-DGGE	NM	(Xu et al., 2011)
Paenalcaligenes	Tianjin Duliu aged vinegar	Acetic acid fermentation	PCR-DGGE/ 454-Pyrosequencing	No	(Peng et al., 2015)
Aurantimonas	Tianjin Duliu aged vinegar	Beginning and middle	PCR-DGGE/ 454-Pyrosequencing	No	(Peng et al., 2015)
Swaminathania	Tianjin Duliu aged vinegar	All stages	PCR-DGGE/ 454-Pyrosequencing	No	(Peng et al., 2015)
Allobaculum stercoricanis	Chinese cereal vinegar	Alcohol and acetic acid fermentation	Illumina MiSeq	No	(Zhang et al., 2020)
Pedobacter	Shanxi aged vinegar	Early stage (days 0-4)	PCR-DGGE/ Pyrosequencing	No	(Zhu et al., 2018)
Saccharopolyspora	Shanxi aged vinegar	Early stage (days 0-4)	PCR-DGGE/ Pyrosequencing	No	(Zhu et al., 2018)
Kroppenstedtia	Shanxi aged vinegar	Later stage (days 22–26)	PCR-DGGE/ Pyrosequencing	Yes	(Zhu et al., 2018)
Exiguobacterium	Rice vinegar	Early stage	PCR-DGGE	No	(Haruta et al., 2006)
Arthrobacter	Zhenjiang aromatic vinegar	NM	Illumina MiSeq/ qPCR	No	(Wang et al., 2016)
Vibrio	Tianjin Duliu aged vinegar	Middle stage	PCR-DGGE/ 454-Pyrosequencing	No	(Peng et al., 2015)

Enhydrobacter	Zhenjiang aromatic vinegar	NM	Illumina MiSeq/ qPCR	No	(Wang et al., 2016)
Oceanimonas	Tianjin Duliu aged vinegar	Middle stage	PCR-DGGE/ 454-Pyrosequencing	No	(Peng et al., 2015)

NM: Not mentioned; DHPLC: Denaturing high performance liquid chromatography; MALDI-TOF MS: Matrix-assisted laser desorption ionization–time-of-flight mass spectrometry; PCR: Polymerase chain reaction; DGGE: Denaturing Gradient Gel Electrophoresis; RFLP: Restriction Fragment Length Polymorphism; ERIC-PCR: Enterobacterial Repetitive Intergenic Consensus Polymerase Chain Reaction; qPCR: quantitative PCR.

Furthermore, vinegar worldwide can be susceptible to contamination from external sources, including opportunistic or pathogenic groups associated with human contact (Table 3). Certain groups, namely *Escherichia* and *Shigella,* have been reported to be present in the initial stages or during various phases of the fermentation process. Despite not being dominant, the presence of these microorganisms, even at low levels, can pose a considerable risk (Zhu et al., 2018). They are widely recognized as major contributors to foodborne illnesses on a global scale and have the potential to trigger outbreaks, even with minimal contamination (McMahon et al., 2022). Fortunately, Escherichia appeared during the early period of AAF fermentation and disappeared on the ninth day (Nie et al., 2013). Acetic acid showed the most lethal action on E. coli strain O157:H7, followed by lactic acid (Wang et al., 2021). Other opportunistic groups that probably originated from raw materials are *Pseudomonas, Enterobacter, Enterococcus, Sphingomonas, Rhodococcus, Corynebacteria, Ralstonia, Nocardioidesa,* and *Klebsiella* (Wang et al., 2016; Zhu et al., 2018).

LAB diversity and functional role

Notably, vinegar exhibits a substantially higher diversity of LAB compared to AAB, with LAB being represented by six genera and 26 species and AAB by three genera and ten species (Table 4). Moreover, LAB demonstrates a pervasive presence in almost all types of vinegar, emphasizing their extensive involvement in fermentation (Figure 2). *Lactobacillus* stands out as the predominant group, constituting over 70% of the total LAB population. This group primarily comprises *L. plantarum, L. casei, L. acetotolerans,* and *L. fermentum*. Following closely behind *Lactobacillus*, the *Pediococcus* genus is predominantly represented by *P. acidilactici* and *P. pentosaceus*.

Furthermore, other species like *Weissella confusa*, *Lactococcus lactis*, *Leuconostoc mesenteroides*, and *Oenococcus* sp. contribute to the intricate microbial landscape observed in vinegar production. Different LAB have been observed to dominate at some stage of the fermentation process in various types of vinegar, such as *Lactobacillus* in all except for rice (Haruta et al., 2006) and cereal vinegar (Zhang et al., 2020); *Pediococcus* and *Lactococcus* in Shanxi-aged vinegar (Wu et al., 2012) and Daqu starter (Tang et al., 2019); *Weissella* in Tianjin Duliu vinegar (Nie et al., 2013) and Qishan

vinegar (Gan et al., 2017); *Leuconostoc* in Qishan vinegar (Gan et al., 2017); and *Oenococcus* sp. in apple vinegar (Song et al., 2019; Trček et al., 2016). Nie et al., (2013) showed that LAB presented greater abundance (>70 %) and diversity in acetic acid fermentation of Tianjin Duliu ripened vinegar than AAB by Illumina sequencing.

In general, *Lactobacillus* and *Acetobacter*, respectively, are the predominant groups in vinegar fermentation (Fang et al., 2021; Zhang et al., 2020; Zhu et al., 2018). This pattern seems to be followed regarding location, cited in traditional Chinese and European vinegars (Nie et al., 2017). *Lactobacillus* can be dominant in association with another LAB genus, such as *Oenococcus* sp. in Shanxi-aged vinegar fermentation (Nie et al., 2017; Trček et al., 2016), *Weissella* (Nie et al., 2013) and *Leuconostoc* in Qishan vinegar (Gan et al., 2017), *Lactococcus* in Shanxi-aged vinegar (Zhu et al., 2018); and *Weissella* in Duliu-daqu (Nie et al., 2013).

Lactobacillus exhibits remarkable stability in ethanoic environments, as documented by (Nie et al., 2017) and can survive at low pH conditions (Gan et al., 2017). Consequently, *Lactobacillus* is likely to be prevalent during the initial stage of AF vinegar fermentation, in conjunction with the metabolic activities of *S. cerevisiae* and other yeasts (Haruta et al., 2006). AF occurs in an anoxic environment, suitable for the growth of facultative anaerobes, including yeasts and LAB (Nie et al., 2017). Also, some studies indicate a symbiotic relation between LAB and yeasts, where yeasts provide amino acids and vitamins for lactic acid production, while LAB provide energy sources (Chen et al., 2017). In this way, LAB are mainly present in the early and middle stages of vinegar fermentation, preceding acid acetic fermentation (AAF) (Zhang et al., 2020).

Throughout the fermentation process, the levels of LAB and other bacteria tend to gradually decrease until the onset of AAF (Zhu et al., 2018). Both sequencing and culture-dependent analysis revealed that *Lactobacillus* dominated the initial stage (day 10 to 30) of Zhejiang rosy vinegar fermentation, while *Acetobacter* remained highly abundant from day 40 until the completion of the process (Fang et al., 2021). *Lactobacillus* exhibited positive correlations with most bacterial genera present in this vinegar, whereas it displayed a negative correlation with *Acetobacter*.

Table 3. Opportunistic pathogenic bacteria present in the vinegar matrix.

Human opportunistic or pathogenic genus/species	Vinegar type	Fermentation stage	Sequencing method	Dominance (Yes/No/Constant)	References
Serratia	Tianjin Duliu aged vinegar	Acetic acid fermentation	PCR-DGGE/ 454-Pyrosequencing	Yes	(Peng et al., 2015)
Escherichia/Shigella	Vinegar Pei	NM	Pyrosequencing	No	(Wang et al., 2015)
Escherichia/Shigella	Qishan vinegar	Early stages	q qPCR/ Illumina HiSeq	No	(Gan et al., 2017)
Escherichia	Apple vinegar	All stages	Illumina MiSeq	Constant	(Song et al., 2019)
Escherichia	Tianjin duliu vinegar	Early stages	PCR-DGGE/ Illumina	No	(Nie et al., 2013)
Escherichia	Daqu starter	All stages	Illumina HiSeq	No	(Tang et al., 2019)
Escherichia	Daqu starter	NM	PCR-DGGE	No	(Li et al., 2019)
Shigella	Apple vinegar	All stages	Illumina MiSeq	Constant	(Song et al., 2019)
Streptococcus	Zhejiang rosy vinegar	Early alcoholic fermentation	llumina MiSeq	No	(Fang et al., 2021)
	Vinegar Pei	Alcoholic fermentation	Pyrosequencing	No	(Wang et al., 2015)

	Tianjin Duliu aged vinegar	Beginning and middle	PCR-DGGE/ 454-Pyrosequencing	No	(Peng et al., 2015)
Pseudomonas	Zhenjiang aromatic vinegar	All stages	Illumina MiSeq/ qPCR	No	(Wang et al., 2016)
	Zhejiang rosy vinegar	All stages	llumina MiSeq	Constant	(Fang et al., 2021)
	Chinese cereal vinegar	Alcohol and acetic acid fermentation	Illumina MiSeq	No	(Zhang et al., 2020)
	Tianjin duliu vinegar	Acid acetic fermentation	PCR-DGGE/ Illumina	No	(Nie et al., 2013)
	Zhenjiang aromatic vinegar	Acid acetic fermentation	PCR-DGGE	NM	(Xu et al., 2011)
	Shanxi aged vinegar	Early stage (days 0-4)	PCR-DGGE/ Pyrosequencing	No	(Zhu et al., 2018)
	Qishan vinegar	Early stages	qPCR/ Illumina HiSeq	No	(Gan et al., 2017)
	Tianjin Duliu aged vinegar	All stages	PCR-DGGE/ 454-Pyrosequencing	No	(Peng et al., 2015)
	Daqu starter	All stages	Illumina HiSeq	No	(Tang et al., 2019)
Pseudomonas cissicola	Zhenjiang aromatic vinegar	Acid acetic fermentation	PCR-DGGE	NM	(Xu et al., 2011)
Staphylococcus	Zhenjiang aromatic vinegar	NM	Illumina MiSeq/ qPCR	No	(Wang et al., 2016)
	Zhenjiang aromatic vinegar	Acid acetic fermentation	PCR-DGGE	NM	(Xu et al., 2011)

	Daqu starter	All stages	Illumina HiSeq	No	(Tang et al., 2019)
Staphylococcus gallinarum	Zhenjiang aromatic vinegar	Acid acetic fermentation	PCR-DGGE	NM	(Xu et al., 2011)
Staphylococcus kloosii	Zhenjiang aromatic vinegar	Acid acetic fermentation	PCR-DGGE	NM	(Xu et al., 2011)
Enterobacter	Tianjin duliu vinegar	Early stages	PCR-DGGE/ Illumina	No	(Nie et al., 2013)
	Zhenjiang aromatic vinegar	Acid acetic fermentation	PCR-DGGE	NM	(Xu et al., 2011)
	Daqu starter	NM	PCR-DGGE	No	(Li et al., 2019)
Enterobacter cloaceae	Daqu starter	NM	PCR-DGGE	Yes	(Li et al., 2019)
Enterobacter hormaechei	Daqu starter	NM	PCR-DGGE	No	(Li et al., 2019)
Enterobacter tabaci	Chinese cereal vinegar	Alcohol and acetic acid fermentation	Illumina MiSeq	No	(Zhang et al., 2020)
Enterococcus	Tianjin Duliu aged vinegar	Beginning and middle	PCR-DGGE/ 454-Pyrosequencing	No	(Peng et al., 2015)
	Zhenjiang aromatic vinegar	Later stage of acetic acid fermentation (days 10–18)	Illumina MiSeq/ qPCR	No	(Wang et al., 2016)
	Zhejiang rosy vinegar	All stages	llumina MiSeq	Constant	(Fang et al., 2021)
Sphingomonas	Tianjin Duliu aged vinegar	All stages	PCR-DGGE/ 454-Pyrosequencing	No	(Peng et al., 2015)

	Zhenjiang aromatic vinegar	All stages	Illumina MiSeq/ qPCR	No	(Wang et al., 2016)
Cronobacter	Daqu starter	NM	PCR-DGGE	No	(Li et al., 2019)
Cronobacter sakazakii	Daqu starter	NM	PCR-DGGE	Yes	(Li et al., 2019)
Rhodococcus	Slovene apple vinegar	Early stages	DHPLC/ Illumina MiSeq	No	(Trček et al., 2016)
	Shanxi aged vinegar	Later stage (days 22–26)	PCR-DGGE/ Pyrosequencing	Yes	(Zhu et al., 2018)
Klebsiella	Tianjin duliu vinegar	NM	PCR-DGGE/ Illumina	No	(Nie et al., 2013)
Corynebacteria	Zhejiang rosy vinegar	Early alcoholic fermentation	llumina MiSeq	No	(Fang et al., 2021)
Corynebacterium	Tianjin Duliu aged vinegar	Middle stage	PCR-DGGE/ 454-Pyrosequencing	No	(Peng et al., 2015)
Achromobacter	Chinese cereal vinegar	Alcohol and acetic acid fermentation	Illumina MiSeq	No	(Zhang et al., 2020)
Helicobacter	Chinese cereal vinegar	Alcohol and acetic acid fermentation	Illumina MiSeq	No	(Zhang et al., 2020)
Nocardioides	Tianjin duliu vinegar	Early stages	PCR-DGGE/ Illumina	No	(Nie et al., 2013)
Oligella	Tianjin Duliu aged vinegar	Acetic acid fermentation	PCR-DGGE/ 454-Pyrosequencing	No	(Peng et al., 2015)
Fusobacterium	Tianjin Duliu aged vinegar	Acetic acid fermentation	PCR-DGGE/ 454-Pyrosequencing	No	(Peng et al., 2015)

Arcobacter	Tianjin Duliu aged vinegar	Final	PCR-DGGE/ 454-Pyrosequencing	No	(Peng et al., 2015)
Roseomonas	Tianjin Duliu aged vinegar	Beginning and middle	PCR-DGGE/ 454-Pyrosequencing	No	(Peng et al., 2015)
Aureimonas	Shanxi aged vinegar	Early stage (0-4 days)	PCR-DGGE/ Pyrosequencing	No	(Zhu et al., 2018)
Ralstonia	Shanxi aged vinegar	All stages	PCR-DGGE/ Pyrosequencing	Yes	(Zhu et al., 2018)
Stenotrophomonas	Zhenjiang aromatic vinegar	NM	Illumina MiSeq/ qPCR	No	(Wang et al., 2016)
Sphingobacterium	Zhenjiang aromatic vinegar	NM	Illumina MiSeq/ qPCR	No	(Wang et al., 2016)
Roseomonas	Zhenjiang aromatic vinegar	NM	Illumina MiSeq/ qPCR	No	(Wang et al., 2016)
Treponema	Tianjin Duliu aged vinegar	Middle stage	PCR-DGGE/ 454-Pyrosequencing	No	(Peng et al., 2015)
Oligella	Tianjin Duliu aged vinegar	Middle stage	PCR-DGGE/ 454-Pyrosequencing	No	(Peng et al., 2015)
Microbacterium	Tianjin Duliu aged vinegar	Middle stage	PCR-DGGE/ 454-Pyrosequencing	No	(Peng et al., 2015)
Comamonas	Tianjin Duliu aged vinegar	Middle stage	PCR-DGGE/ 454-Pyrosequencing	No	(Peng et al., 2015)

NM: Not mentioned; DHPLC: Denaturing high performance liquid chromatography; MALDI-TOF MS: Matrix-assisted laser desorption ionization–time-of-flight mass spectrometry; PCR: Polymerase chain reaction; DGGE: Denaturing Gradient Gel Electrophoresis; RFLP: Restriction Fragment Length Polymorphism; ERIC-PCR: Enterobacterial Repetitive Intergenic Consensus Polymerase Chain Reaction; qPCR: quantitative PCR.

As fermentation progresses, *Acetobacter* actively generates a significant quantity of acetic acid, increasing titratable acid content. This rise in acidity contributes to the exclusion of other bacterial genera suggesting co-exclusion relations between *Acetobacter* and most genera (Fang et al., 2021). Early-stage groups, including LAB, exhibited a disappearance by the ninth day of AAF in Tianjin Duliu vinegar fermentation, indicating a low tolerance to acetic acid (Nie et al., 2013). In the process of Zhejiang rosy vinegar, *Lactobacillus* displayed a continuous decline and ceased to be the dominant bacteria during the middle and later phases of fermentation (Fang et al., 2021). The relative abundance of LAB reaches >90% during AF of Shanxi-aged vinegar and decreases to 49% at the end of AAF (Nie et al., 2017). Nevertheless, the detection of certain LAB strains in AF and AAF suggests the possibility of domestication within the genus (Nie et al., 2017). Some persistent species are *L. acetotolerans* (Wu et al., 2021), *Companilactobacillus alimentarius* (former *Lactobacillus alimentarius*) and *Limosilactobacillus reuteri* (former *Lactobacillus reuteri*) in Chinese cereal vinegar (Haruta et al., 2006), *L. fermentum* in Zhejiang rosy vinegar (Fang et al., 2021), *Limosilactobacillus panis* (former *Lactobacillus panis*) in Zhenjiang aromatic vinegar, and *L. helveticus* in Tianjin Duliu vinegar and Chinese cereal vinegar. *P. acidilactici* and *W. confusa* were also persistent in Chinese cereal vinegar during AAF. LAB frequently faces acidic matrices in fermented foods. To survive they developed innumerous mechanisms to increase their resistance in hostile environments. These regulations in the metabolism pathways involve mainly proton pump, cell membrane composition changes, cell density, genetic material and protein repair and acid neutralization processes (production of alkaline substances such as arginine, urea, and ammonia) (Wang et al., 2018).

Some physicochemical factors can influence LAB performance in vinegar fermentation. Oxygen levels and temperature are strongly correlated with *L. acetotolerans*, *L. alimentarius,* and *L. helveticus*, as well as the AAB *A. pasteurianus* and *Komagataeibacter* (Zhang et al., 2020). 30◦C and 40◦C are the optimal temperatures for AAB and LAB, respectively. *Lactobacillus* is a temperature-sensitive genus and decreases or even disappears under elevated temperature. Thus, *Acetobacter*'s temperature-tolerant genus is self-screened and dominates the production of acids (Wu et al., 2021). In this way, their population may be quite controlled. For example, vinegar Pei

is stirred manually, resulting in more LAB from the bottom to the upper layer, producing significant amounts of lactic acid, amino acids and other flavor compounds during vinegar fermentation (Nie et al., 2013).

LAB plays a pivotal role as the primary producer of lactic acid through organic acid metabolism, owing to its positive correlation with this acid. Conversely, *Komagataeibacter* and *Acetobacter* are positively associated with acetic acid production (Wu et al., 2021; Zhu et al., 2018). Furthermore, LAB exhibits the ability to metabolize sugars via two distinct pathways: as either homofermentative or heterofermentative bacteria. Depending on the environmental conditions, heterofermentative species ferment sugars and produce not only lactic acid but also acetic acid, resembling the metabolic characteristics of AAB. During initial AAF, ethanol was the primary molecule available, and genes ackA and ldh were highly expressed in *Lactobacillus*, suggesting lactic and acetic acid metabolism. On the third day of Chinese cereal vinegar, the heat resulting from fermentation inhibits this gene expression (Wu et al., 2021). *Acetobacter* appeared on the fifth day and combined with *Lactobacillus*, produced acetic acid. Lactic acid then decreased, probably due to the re-conversion of lactic acid under high oxygen conditions (Wu et al., 2021). In Cupei vinegar, *Lactobacillus* also plays a significant role in organic acid metabolism at initial AAF due to its high abundance (Nie et al., 2017).

Table 4. Vinegar LAB diversity.

BAL genus/species	Vinegar type	Fermentation stage	Sequencing method	Dominance (Yes/No/Constant)	References
Lactobacillus	Apple vinegar	Alcoholic fermentation	Illumina MiSeq	Yes	(Song et al., 2019)
	Slovene apple vinegar	Alcoholic fermentation	DHPLC/ Illumina MiSeq	Yes	(Trček et al., 2016)
	Shanxi aged vinegar	Medium stage (days 5–21)	PCR-DGGE/ Pyrosequencing	Yes	(Zhu et al., 2018)
	Rice vinegar	Early stage	PCR-DGGE	No	(Haruta et al., 2006)
	Zhejiang rosy vinegar	Early alcoholic fermentation	llumina MiSeq	Yes	(Fang et al., 2021)
	Vinegar Pei	Alcoholic fermentation	Pyrosequencing	Yes	(Wang et al., 2015)
	Chinese cereal vinegar	Alcohol and acetic acid fermentation	Illumina MiSeq	No	(Zhang et al., 2020)
	Tianjin duliu vinegar	Acetic acid fermentation	PCR-DGGE/ Illumina	Yes	(Nie et al., 2013)
	Zhenjiang aromatic vinegar	Acid acetic fermentation	PCR-DGGE	NM	(Xu et al., 2011)
	Qishan vinegar	Acetic acid fermentation	qPCR/ Illumina HiSeq	Yes	(Gan et al., 2017)

	Tianjin Duliu aged vinegar	Acetic acid fermentation	PCR-DGGE/ 454-Pyrosequencing	Yes	(Peng et al., 2015)
	Daqu starter	All stages	Illumina HiSeq	Yes	(Tang et al., 2019)
	Zhenjiang aromatic vinegar	Early stage of acid acetic fermentation (days 0–9)	Illumina MiSeq/ qPCR	Yes	(Wang et al., 2016)
Lactobacillus plantarum	Apple vinegar	Alcoholic fermentation	Illumina MiSeq	No	(Song et al., 2019)
	Rice vinegar	Early stage	PCR-DGGE	No	(Haruta et al., 2006)
	Apple-based kefir vinegar	All stages	MALDI-TOF MS	Constant	(Viana et al., 2017)
	Tianjin duliu vinegar	Early stages	PCR-DGGE/ Illumina	No	(Nie et al., 2013)
	Shanxi aged vinegar	Alcoholic fermentation	Inter-delta PCR/ PCR-RFLP/ ERIC-PCR/ 16S rRNA and 26S rRNA partial gene sequencing	Yes	(Wu et al., 2012)
Lactobacillus casei	Apple vinegar	Alcoholic fermentation	Illumina MiSeq	No	(Song et al., 2019)
	Rice vinegar	Early stage	PCR-DGGE	No	(Haruta et al., 2006)
	Meigui Rice Vinegar	NM	PCR-DGGE	No	(Shi et al., 2013)
	Shanxi aged vinegar	Alcoholic fermentation	Inter-delta PCR/ PCR-RFLP/ ERIC-PCR/ 16S	Yes	(Wu et al., 2012)

			rRNA and 26S rRNA partial gene sequencing		
Lactobacillus acetotolerans	Rice vinegar	Early stage	PCR-DGGE	Yes	(Haruta et al., 2006)
	Chinese cereal vinegar	Alcohol and acetic acid fermentation	Illumina MiSeq	Yes	(Zhang et al., 2020)
	Tianjin duliu vinegar	Early stages	PCR-DGGE/ Illumina	No	(Nie et al., 2013)
Lactobacillus fermentum	Rice vinegar	Later stage	PCR-DGGE	No	(Haruta et al., 2006)
	Shanxi aged vinegar	Alcoholic fermentation	Inter-delta PCR/ PCR-RFLP/ ERIC-PCR/ 16S rRNA and 26S rRNA partial gene sequencing	Yes	(Wu et al., 2012)
	Zhejiang rosy vinegar	Alcoholic fermentation	llumina MiSeq	Yes	(Fang et al., 2021)
Lactobacillus panis	Zhenjiang aromatic vinegar	Acid acetic fermentation	PCR-DGGE	NM	(Xu et al., 2011)
	Chinese cereal vinegar	Alcohol and acetic acid fermentation	Illumina MiSeq	No	(Zhang et al., 2020)
	Tianjin duliu vinegar	Early stages	PCR-DGGE/ Illumina	No	(Nie et al., 2013)
Lactobacillus alimentarius	Tianjin duliu vinegar	Early stages	PCR-DGGE/ Illumina	No	(Nie et al., 2013)
	Chinese cereal vinegar	Alcohol and acetic acid fermentation	Illumina MiSeq	No	(Zhang et al., 2020)

Lactobacillus reuteri	Meigui Rice Vinegar	NM	PCR-DGGE	No	(Shi et al., 2013)
	Chinese cereal vinegar	Alcohol and acetic acid fermentation	Illumina MiSeq	No	(Zhang et al., 2020)
Lactobacillus helveticus	Tianjin duliu vinegar	Acid acetic fermentation	PCR-DGGE/ Illumina	No	(Nie et al., 2013)
	Chinese cereal vinegar	Alcohol and acetic acid fermentation	Illumina MiSeq	Yes	(Zhang et al., 2020)
Lactobacillus paracasei	Apple-based kefir vinegar	All stages	MALDI-TOF MS	Constant	(Viana et al., 2017)
	Rice vinegar	Early stage	PCR-DGGE	No	(Haruta et al., 2006)
Lactobacillus acidophilus	Meigui Rice Vinegar	NM	PCR-DGGE	No	(Shi et al., 2013)
Lactobacillus brevis	Rice vinegar	Early stage	PCR-DGGE	No	(Haruta et al., 2006)
Lactobacillus buchneri	Shanxi aged vinegar	Alcoholic fermentation	Inter-delta PCR/ PCR-RFLP/ ERIC-PCR/ 16S rRNA and 26S rRNA partial gene sequencing	Yes	(Wu et al., 2012)
Lactobacillus salivarius	Apple vinegar	Alcoholic fermentation	Illumina MiSeq	No	(Song et al., 2019)
Lactobacillus farraginis	Apple vinegar	Alcoholic fermentation	Illumina MiSeq	No	(Song et al., 2019)
Lactobacillus delbrueckii subsp. bulgaricus	Meigui Rice Vinegar	Final stage	PCR-DGGE	Yes/constant	(Shi et al., 2013)

Lactobacillus pontis	Tianjin duliu vinegar	Early stages	PCR-DGGE/ Illumina	No	(Nie et al., 2013)
Lactobacillus ruminis	Tianjin duliu vinegar	Early stages	PCR-DGGE/ Illumina	No	(Nie et al., 2013)
Lactococcus garvieae	Tianjin duliu vinegar	Early stages	PCR-DGGE / Illumina	No	(Nie et al., 2013)
Lactobacillus homohiochii	Tianjin duliu vinegar	Early stages	PCR-DGGE/ Illumina	No	(Nie et al., 2013)
Lactobacillus gallinarum	Zhenjiang aromatic vinegar	Early stage	PCR-DGGE	NM	(Xu et al., 2011)
Pediococcus	Shanxi aged vinegar	Early stage (0-4 days)	PCR-DGGE/ Pyrosequencing	Yes	(Zhu et al., 2018)
	Tianjin duliu vinegar	Early stages	PCR-DGGE/ Illumina	No	(Nie et al., 2013)
	Daqu starter	NM	PCR-DGGE	No	(Li et al., 2019)
	Tianjin Duliu aged vinegar	All stages	PCR-DGGE/ 454-Pyrosequencing	No	(Peng et al., 2015)
	Daqu starter	All stages	Illumina HiSeq	Yes	(Tang et al., 2019)
Pediococcus acidilactici	Rice vinegar	Early stage	PCR-DGGE	No	(Haruta et al., 2006)
	Chinese cereal vinegar	Alcohol and acetic acid fermentation	Illumina MiSeq	No	(Zhang et al., 2020)
	Shanxi aged vinegar	Alcoholic fermentation	Inter-delta PCR/ PCR-RFLP/ ERIC-PCR/ 16S	Yes	(Wu et al., 2012)

			rRNA and 26S rRNA partial gene sequencing		
Pediococcus pentosaceus	Shanxi aged vinegar	Alcoholic fermentation	Inter-delta PCR/ PCR-RFLP/ ERIC-PCR/ 16S rRNA and 26S rRNA partial gene sequencing	Yes	(Wu et al., 2012)
	Daqu starter	NM	PCR-DGGE	Yes	(Li et al., 2019)
Weissella	Rice vinegar	Early stage	PCR-DGGE	No	(Haruta et al., 2006)
	Zhejiang rosy vinegar	Early alcoholic fermentation	llumina MiSeq	No	(Fang et al., 2021)
	Tianjin duliu vinegar	Early stages	PCR-DGGE/ Illumina	Yes	(Nie et al., 2013)
	Daqu starter	NM	PCR-DGGE	No	(Li et al., 2019)
	Tianjin Duliu aged vinegar	Beginning and middle	PCR-DGGE/ 454-Pyrosequencing	No	(Peng et al., 2015)
	Zhenjiang aromatic vinegar	NM	Illumina MiSeq/ qPCR	No	(Wang et al., 2016)
	Qishan vinegar	Acetic acid fermentation	qPCR/ Illumina HiSeq	Yes	(Gan et al., 2017)
Weissella confusa	Chinese cereal vinegar	Alcohol and acetic acid fermentation	Illumina MiSeq	No	(Zhang et al., 2020)
	Tianjin duliu mature vinegar	Early stages	PCR-DGGE/ Illumina	No	(Nie et al., 2013)

	Shanxi aged vinegar	Alcoholic fermentation	Inter-delta PCR/ PCR-RFLP/ ERIC-PCR/ 16S rRNA and 26S rRNA partial gene sequencing	Yes	(Wu et al., 2012)
Lactococcus	Zhenjiang aromatic vinegar	Later stage of acetic acid fermentation (days 10–18)	Illumina MiSeq/ qPCR	No	(Wang et al., 2016)
	Daqu starter	All stages	Illumina HiSeq	No	(Tang et al., 2019)
	Apple vinegar	Acetic acid fermentation	Illumina MiSeq	Yes	(Song et al., 2019)
	Tianjin duliu vinegar	Early stages	PCR-DGGE/ Illumina	Yes	(Nie et al., 2013)
	Shanxi aged vinegar	Early stage (0-4 days)	PCR-DGGE/ Pyrosequencing	Yes	(Zhu et al., 2018)
Lactococcus lactis	Rice vinegar	Early stage	PCR-DGGE	No	(Haruta et al., 2006)
Leuconostoc	Vinegar Pei	Beginning/ Alcoholic fermentation	Pyrosequencing	No	(Wang et al., 2015)
	Qishan vinegar	Acetic acid fermentation	qPCR/ Illumina HiSeq	Yes	(Gan et al., 2017)
	Daqu starter	All stages	Illumina HiSeq	Yes	(Tang et al., 2019)
Leuconostoc mesenteroides subsp mesenteroides	Meigui Rice Vinegar	NM	PCR-DGGE	No	(Shi et al., 2013)
Oenococcus	Apple vinegar	Alcoholic fermentation	Illumina MiSeq	Yes	(Song et al., 2019)

	Slovene apple vinegar	Alcoholic fermentation	DHPLC/ Illumina MiSeq	Yes	(Trček et al., 2016)

NM: Not mentioned; DHPLC: Denaturing high performance liquid chromatography; MALDI-TOF MS: Matrix-assisted laser desorption ionization–time-of-flight mass spectrometry; PCR: Polymerase chain reaction; DGGE: Denaturing Gradient Gel Electrophoresis; RFLP: Restriction Fragment Length Polymorphism; ERIC-PCR: Enterobacterial Repetitive Intergenic Consensus Polymerase Chain Reaction; qPCR: quantitative PCR.

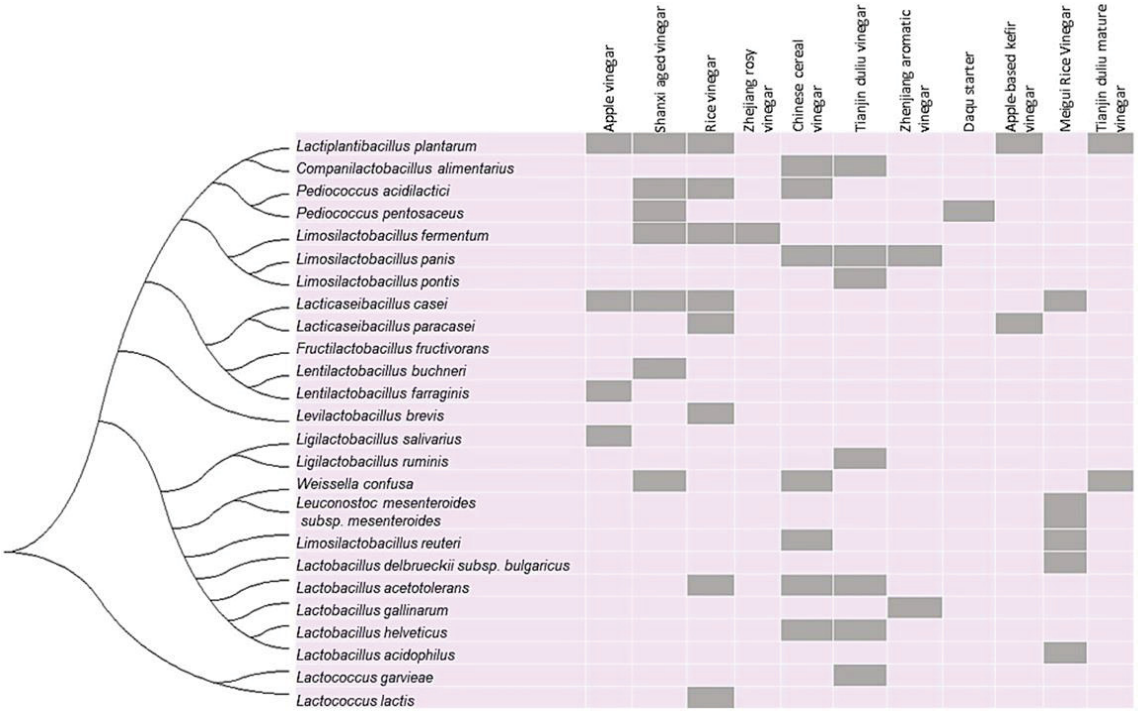


Figure 2. 16S rRNA Neighbor-joining tree showing the phylogenetic proximity of LAB species reported in different types of vinegar. The 16S rRNA gene sequences were retrieved from the GenBank database and aligned with ClustalW. The phylogenetic tree was constructed using the MEGA X program. Data was collected from work by Song et al. (2019), Haruta et al. (2006), Viana et al. (2017), Nie et al. (2013), Wu et al. (2012), Shi et al. (2013), Zhang et al. (2020), Nie et al. (2013), Fang et al. (2021), Xu et al. (2011), Viana et al. (2017), Zhu et al. (2018), Li et al. (2019), Peng et al. (2015), Tang et al. (2019), Gan et al. (2017) and Wang et al. (2016).

LAB notably impacts natural vinegar fermentation through sensorial improvement, safety (Chai et al., 2020a; Nie et al., 2013), and quality of the final product (Chen et al., 2017). Lactic acid is the second most prominent organic acid, mainly produced from the early to middle-early stage (Zhang et al., 2020). It contributes to the fresh and sour taste of vinegar, softening the strong flavor of the condiment (Nie et al., 2017). Lactic acid is the primary non-volatile acid and can mitigate the intense flavor and taste of vinegar, offering a more palatable experience for certain individuals (Wang et al., 2016). *Lactobacillus* showed significantly positive correlations with dodecanoic acid, ethyl ester, and 2,4- di-tert-butylphenol (Fang et al., 2021), rising cheesy flavor. The bacterial species most related to non-volatile acids in Chinese cereal vinegar comprise *Lentilactobacillus acetotolerans* (formerly *Lactobacillus acetotolerans*) and *Lacticaseibacillus helveticus* (formerly *Lactobacillus helveticus*), which accounted for >85 % of LAB (Zhang et al., 2020). It was found that the content of benzaldehyde gradually augmented with the increasing abundance of *Lactobacillus* in Shanxi-aged

vinegar, suggesting that it plays an important role in forming almond flavor (Zhu et al., 2018). *Lactococcus* and *Pediococcus* dominance in the early stage represented a positive correlation to esters including ethyl acetate, hexanoic acid, ethyl ester, propanoic acid, 2-hydroxy-ethyl ester, ethanol, and 3-methyl-1-butanol, which suggests that these bacteria participate in esterification, providing important effect for the aroma of Shanxi-aged vinegar in the early stage. Also, *Lactobacillus* has a positive relation which contributed to the content of the propanoic acid-2-hydroxy-ethyl ester, rising aroma of alcohol, fruit, and cream (Zhu et al., 2018). Additionally, *Lactococcus* has a positive correlation to 2-octanone which can add desirable fruity, blue and parmesan cheese notes with mushroom and dairy nuances to the final product (Zhu et al., 2018).

During solid-state fermentation of cereal vinegar, acetoin is an important flavor substance, releasing a creamy yogurt aroma and buttery taste. *Lacticaseibacillus casei* and *Acetobacter pasteurianus* enhance acetoin accumulation, key contributors to vinegar aroma (Chai et al., 2020a). Many LAB species are associated with acetoin metabolism, such as *Lactococcus lactis*, *Lacticaseibacillus rhamnosus*, *Lactobacillus crustorum*, *Lactobacillus flora*, and *Lacticaseibacillus casei*. It was found that *Acetobacter* strains in co-culture with *L. fermentum* had greater efficiency in acetoin accumulation than *Acetobacter* in monocultures (Zhao and Yun, 2016).

During the initial stages of fermentation, LAB effectively inhibits the proliferation of undesirable environmental microorganisms. This is due to the potential antimicrobial activity of lactic acid, which lowers the pH of the fermentation matrices. Additionally, LAB's production of bacteriocins is a noteworthy function in food processing, as these compounds effectively inhibit the growth of spoilage and pathogenic microorganisms (Fang et al., 2021; Haruta et al., 2006). In traditional Shanxi-aged vinegar, lactic acid demonstrates inhibitory effects on various microorganisms, including *Rhizobium, Sphingomonas, Pantoea, Pseudomonas, Methylobacterium,* and *Cladosporium*, as documented by Nie et al., (2017). Furthermore, LAB can act to inhibit harmful molecules formed during vinegar processing. Free amino groups and reducing sugars found in liquid-state vinegar fermentation can react with each other and form advanced glycation end-products (AGEs). Absorption of these molecules is known to cause accelerated aging and diabetic complications such as inflammation, nephropathy, protein denaturation and oxidative stress (Li et al., 2022). Then, exogenous chemical inhibitors, such as catechin,

phenolics, and alkaloids, are recommended; however, the high cost and potential adverse effects of these inhibitors difficult their industrial use. *Limosilactobacillus fermentum* showed a great inhibitory effect on the formation of different AGEs in fermentation, contributing to the safety of the final product. Moreover, *Limosilactobacillus fermentum* improved the variety of flavor compounds, including esters, alcohols, phenols and acids (Li et al., 2022).

Metabolites and aromas secreted by LAB

Taste (sweet, bitter, sour, salty, and umami) and aroma are the key attributes that define the quality and degree of acceptance of vinegar by consumers. It is known that the sensory profile of these foods is mainly shaped by organic acids that include acetic, lactic, tartaric, citric acid, and volatile compounds such as esters, aldehydes, ketones, alcohols, and terpenes. In addition, these metabolites also have a significant impact on the appearance and texture of the final product (Hu et al., 2022; Peyer et al., 2016). Although the precursors required for the formation of these aromatic compounds are diverse, the main substrates that can be used by the microbiota involved in vinegar production are carbohydrates, proteins, fatty acids, and citric acid (Smid and Kleerebezem, 2014). However, the proportion of these components can vary significantly between the raw materials used in vinegar manufacturing, resulting in a great diversity of flavors as observed in Figure 2 (Ji, 2022).

Figure 3 illustrates the primary substrates and metabolic pathways employed by LAB during vinegar fermentation. The fermentation of vinegar is a complex process that produces acetic acid as the main acid (Xia et al., 2020). Lactic acid, tartaric acid, and citric acid are non- volatile acids that can also be present in vinegar, although in varying concentrations depending on the specific fermentation conditions and raw materials used (Raspor et al., 2008). While acetic and lactic acids are derived from microbial metabolism, tartaric and citric are organic acids commonly found in fruits and are often present in the raw materials used for vinegar production (Jayabalan et al., 2014). The metabolic pathway of AAB involved in acetic acid production is called the “oxidative pathway” or the “Krebs cycle bypass pathway”. This pathway allows AAB to efficiently convert ethanol to acetic acid, resulting in vinegar's characteristic sour taste and acidity (Mamlouk and Gullo, 2013; Trček et al., 2016). On the other hand, LAB can contribute to acetic acid production through the phosphoketolase pathway. The phosphoketolase

pathway produces three key vinegar's characteristic sour taste and acidity metabolic end products: lactic acid, acetic acid, and carbon dioxide, with ethanol possibly being produced as a minor product (Gullo et al., 2014).

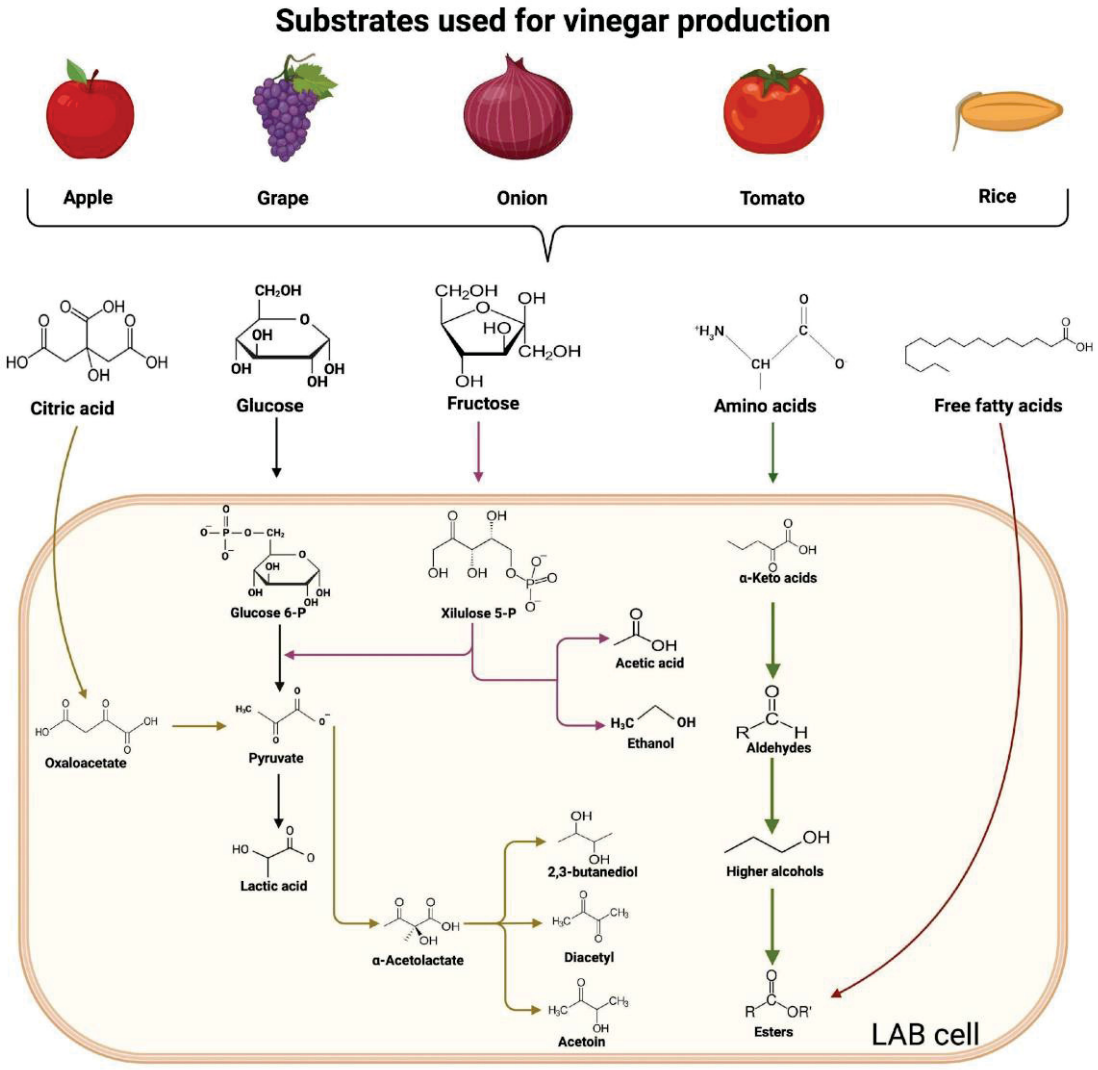


Figure 3. Schematic representation of the major metabolic pathways and metabolites generated by LAB during vinegar fermentation from existing precursors of main substrates.

In general, lactic acid is the second most prevalent organic acid in the final vinegar product, with a wide range from 2575.7 to 30,336.6 mg/L (Fang et al., 2021; Nie et al., 2013; Xia et al., 2020; Zhu et al., 2016). This indicates variations in fermentation conditions or the presence of other microorganisms capable of producing lactic acid. LAB are the main producer of lactic acid. Generally, these microorganisms use a metabolic pathway called Embden-Meyerhof-Parnas (EMP) to convert glucose to pyruvate, which is transformed into lactic acid by lactate dehydrogenase (de Melo Pereira et al., 2020a;

Endo et al., 2014). Among LAB, species belonging to the *Lactobacillus* genus are often associated with lactic acid production in vinegar (Li et al., 2016; Zhu et al., 2018). However, a study by Wang et al., (2016) showed that although *Lactobacillus* dominated the fermentation, variations in lactic acid content during the fermentation process also showed a positive correlation with *Weissella*.

Regarding the volatile fraction, it is known that not all compounds contribute to the flavor and aroma of the product; however, the major metabolites have a substantial effect on flavor characteristics (Wang et al., 2019). The formation of volatile compounds during traditional vinegar production and the impact on the final product is still poorly explored, but recent studies have shown that >60 aromatic compounds have been identified (Fang et al., 2021; W. Wang et al., 2023; Zhu et al., 2016, 2018). In general, more molecules belonging to esters and to a lesser extent, alcohols, aldehydes, and ketones have been observed (Fang et al., 2021; W. Wang et al., 2023). For example, 26 esters, six alcohol, five aldehydes, and four ketones were identified in Pei of Shanxi-aged vinegar (Zhu et al., 2018). This same profile was also detected in Zhejiang rosy vinegar, with ethyl acetate being the main ester identified in both studies (Fang et al., 2021; Zhu et al., 2018). Other molecules, such as propanoic acid-2-hydroxy-ethyl ester and hexanoic acid ethyl ester, were also detected and positively correlated with *Lactococcus* and *Lactobacillus* (Zhu et al., 2018). These compounds can generally be generated from the catabolism of amino acids performed by LABs. Metabolism in question plays an important role in obtaining energy under nutrient-limited conditions and maintaining pH homeostasis (Mayo et al., 2010). Furthermore, a study by Mutaguchi et al., (2013) showed that LAB metabolism is primarily responsible for increasing the amino acid content in the medium during tomato vinegar fermentation. On the other hand, when LAB is in a rich environment, such as that of vinegar, the surplus amino acids become precursors for a range of flavor active compounds that can be generated by either the transaminases or lyase pathways or by enzymatic conversion (Smid and Kleerebezem, 2014; Smit et al., 2005).

In addition to amino acid catabolism, *Lactobacillus, Pediococcus*, and *Lactococcus* species can convert citric acid to aromatic compounds (De Melo Pereira et al., 2020). This metabolism transports extracellular citric acid into the cytoplasm via membrane-associated permeases. After citrate enters the cell, it is transformed into

oxaloacetate under the catalysis of the citric acid lyase complex. Next, oxaloacetate is decarboxylated by oxaloacetate decarboxylase to produce pyruvate and carbon dioxide (Wang et al., 2021). The acidic environment of vinegar favors the accumulation of pyruvate and the production of α-acetolactate, a precursor of active flavor compounds such as 2,3-butanediol, diacetyl, and acetoin (Sánchez-Zurano et al., 2021; Snoep et al., 1992; Wang et al., 2021).

In recent years, high-throughput sequencing has been applied to understand the behavior of bacteria in traditional vinegar (Tables 1, 2, 3, and 4). However, it is still challenging to correlate these microbial structures with the compounds generated during the fermentative process. Thus, further investigations are still needed to understand the production dynamics of volatile compounds and their relationships with different microbial groups.

Functional properties

Vinegar contains a rich profile of bioactive compounds, including organic acids, polyphenols, melanoidins, tetramethylpyrazine, amino acids, vitamins, and minerals. These compounds have been extensively studied for their potential health benefits (Xia et al., 2020). However, the relationship between the health benefits of LAB and vinegar consumption is often overlooked. Many LAB reported in Table 4 have been characterized as probiotics. In addition, the metabolic by-products or components released by probiotic microorganisms during fermentation have gained attention for their potential health benefits to consumers. These compounds, called postbiotics, comprise bioactive soluble molecules that do not fit traditional definitions of probiotics, prebiotics, or para probiotics and include enzymes, peptides, polysaccharides, and other bioactive compounds (Moradi et al., 2020).

The “Guidelines for Evaluation of Probiotics in Food” published by the Food and Agriculture Organization and World Health Organization set forth safety and effectiveness standards for probiotics. These guidelines propose various criteria for probiotic selection, encompassing resistance to adverse conditions within the human body, epithelial adhesion capability, antimicrobial activity, and safety assessment. There is no comprehensive study on the probiotic potential of LAB contained in vinegar. However, specific studies have pointed out the probiotic potential of different sprains in

isolated cases. In their study, Sui et al., (2021) isolated four strains of *Lactiplantibacillus plantarum* and conducted comprehensive evaluations of several characteristics, including bile salt hydrolysis, resistance to gastrointestinal fluids, antioxidant and antimicrobial activity, haemolytic activity, antibiotic resistance, auto- aggregation, co-aggregation, and adhesion to human Caco-2 cells. Particularly noteworthy, the *L. plantarum* NF4 strain exhibited promising probiotic potential along with a hypolipidemic effect.

P.s acidilactici has been identified in different types of vinegar, such as rice, Chinese cereal and Shanxi-aged vinegar (Table 4). The potential probiotic activity of this species was previously accessed through tolerance to gastrointestinal fluids, auto-aggregation and antimicrobial activity against *Listeria, Salmonella, Enterococcus*, and *Staphylococcus* by Jaiswal et al., (2022). Furthermore, the strain studied presented the ability to adhere to colon cells and as an antiproliferative effect against colon cancer cells.

Lacticaseibacillus helveticus (former *L. helveticus)*, LAB related to Tianjin Duliu and Chinese cereal vinegar microbiota, had its probiotic potential demonstrated in koumiss, a traditional fermented beverage produced with mare's milk (Rong et al., 2015). The authors proposed the main pathways associated with the reduction of blood lipid levels following *koumiss* consumption; a metabolomic approach revealed an increase in stearic acid, butyrate, linoleic acid, sphingosine, alanine, tyrosine, α, and γ-tocotrienol levels, which were related to the hypolipidemic effect. Moreover, *L. helveticus* strain showed tolerance to gastrointestinal fluids, high capacity for adhesion to intestinal cells and self-aggregation.

The species *Levilactobacillus brevis* (former *Lactobacillus brevis*), *L. plantarum*, and *L. fermentum* associated with vinegar (Table 4) were identified as potential probiotics by Angmo et al., (2016). In this study, the probiotic potential of 25 different LAB strains isolated from Indian fermented beverages and foods were evaluated i*n vitro*. A principal component analysis (PCA) was performed to select the ten most promising strains according to the probiotic potential tests (lysozyme tolerance, exopolysaccharide production, and β-galactosidase activity) and choose that one closer to *L. casei Shirota* profile (reference strain). An *L. plantarum* strain was the most promising to apply as a probiotic in fermented beverages.

Although *Lactobacillus* comprises the most abundant genus in vinegar LAB diversity, *Weissella* has been identified in different types of vinegar, such as Chinese cereal, Tianjin Duliu ripened and Shanxi-aged vinegar. The probiotic potential of *Weissella* strains isolated from a traditional Indian fermented food was performed by (S. Sharma et al., 2018). *Weissella confusa* showed lysozyme and bile acid tolerance, auto-aggregation and co-aggregation properties; besides that, antioxidant activity, cholesterol control capacity, inhibition of pathogens biofilm formation, enzymatic activities of proteases and β-galactosidase production were also demonstrated. The authors emphasized the potential of *W. confusa* as a probiotic, highlighting its safety aspects, including susceptibility to antibiotics, absence of haemolytic activity, and absence of DNase and gelatinase activities.

In line with these results, Lakra et al., (2020) also demonstrated the probiotic properties of *W. confusa* and *W. cibaria* strains isolated from a fermented batter (dosa). Considering that one of the most important features of probiotics is the tolerance to stress caused by gastrointestinal fluids and bile salts, both species are considered promising candidates for probiotic use by the food industry. Cai et al., (2022) have linked a mechanism called the HigBA toxin-antitoxin system to the stress response triggered by high concentrations of bile salts. In this system, HigB acts as the toxin protein while HigA functions as the cognate antitoxin protein. During stressful conditions, HigA is hydrolyzed, liberating the HigBA complex from the operator region and, thereby forming stress-persistent cells. Furthermore, overexpressed genes associated with stress response were identified in *Weissella* spp., shedding light on their role in stress adaptation within probiotic strains.

Several other LAB strains with probiotic potential already studied in different types of fermented foods can be correlated with bacteria isolated from vinegar, for example, *Pediococcus pentosaceus* isolated from Idly batter (Vidhyasagar and Jeevaratnam, 2013); *L. buchneri* isolated from kimchi (Cheon et al., 2020); *L. delbrueckii subsp. bulgaricus* isolated from homemade yogurt (Tok and Aslim, 2010); *L. lactis from kimchi* (Lee et al., 2015); *Leu. mesenteroides subsp. mesenteroides* isolated from Brazilian water buffalo mozzarella cheese (de Paula et al., 2014); *O. oeni* e *L. casei* from water kefir grains (Yin et al., 2021).

Probiotic yeasts undergo similar evaluations to probiotic bacteria, including assessments of bile and acid tolerance, auto-aggregation, co- aggregation, and safety. *Saccharomyces* and non-*Saccharomyces* play an essential role during the saccharification and alcoholic fermentation process in vinegar (Wu et al., 2012). Although studies in vinegar have not demonstrated the probiotic effect of yeasts, their safety has been evaluated for industrial uses, with promising results for *S. cerevisiae*, S. *boulardii, Pichia anomala, P. kudriavzevii, Lachancea thermotolerans, Candida vini*, and *Hanseniaspora osmophila* (Fernández-Pacheco et al., 2021a). Additionally, genera such as *Debaryomyces, Meyerozyma*, and *Torulaspora* have also demonstrated probiotic effects for application in fermented foods (Staniszewski and Kordowska-Wiater, 2021). *S. boulardii* is the most studied probiotic yeast. In addition to its high tolerance to gastrointestinal conditions, i.e., surviving in high concentrations of acid and bile salts and low pH values, *S. boulardii* can modulate inflammatory processes, since it reduces the levels of pro-inflammatory molecules (Shruthi et al., 2022). Furthermore, it helps to recover from diarrhea, producing digestive enzymes that play an important role in the host's health, impacting the intestinal absorption of nutrients. *S. boulardii* also participates in quorum sensing pathways, modulating the intestinal microbiota. It can be regarded as a multifunctional probiotic, given its numerous additional positive health effects (Yadav et al., 2019).

Among non-*Saccharomyces* yeasts, several strains of *P. kudriavzevii*, isolated from cocoa in Indonesia, were evaluated for antioxidant capacity and probiotic potential (Wulan et al., 2021). Among all isolates evaluated, ten stood out as promising candidates for this purpose. *Pichia kudriavzevii* strains isolated from African fermented cereal-based foods were assessed for their potential use as probiotics, focusing on their adherence to intestinal Caco-2 cells and their ability to produce folate and phytase. *P. kudriavzevii* demonstrated promising results, enhancing nutritional quality through the production of folate and phytase, in addition to meeting probiotic criteria (Greppi et al., 2017).

Meyerozyma caribbica isolated from pineapple showed satisfactory results in comparison with *S. boulardii* (used as a probiotic control strain), specially related to tolerance to bile, acids and pepsin, autoaggregation, hydrophobicity, and resistance to antibiotics. The last one is particularly interesting for yeasts, as they are more resistant than bacteria. Therefore, antibiotic treatments would not affect the probiotic effect of

yeast (Amorim et al., 2018). *M. caribbica* was also identified by Fernández-Pacheco et al., (2021b) in Brazilian flowers and fruits. Other yeasts, such as *Rhodotorula mucilaginosa* and *Diutina rugosa* were also identified, presenting more promising results when compared to *S. boulardii*.

Most of the studies examined in this review focus on non-vinegar microbial strains possessing probiotic properties. This result shows that this fermented product is poorly explored for future research on possible probiotic use. Although *in vitro* and *in vivo* studies have shown promising results for the strains, human studies are crucial to validate the beneficial effects on human health, being a probiotic or postbiotic. Furthermore, determining the appropriate doses and potential adverse effects associated with the consumption of highly acidic products such as vinegar will undoubtedly contribute to a better understanding of the real health benefits of vinegar consumption. For now, vinegar appears to be a potent postbiotic functional food as it presents numerous corroborations of its intake benefits.

Conclusion

In conclusion, this study has shed light on the intriguing role of vinegar as a reservoir of LAB and its potential as a source of acid- resistant probiotics. Our findings have highlighted the rich diversity of LAB present in vinegar, indicating its suitability for further exploration in the field of probiotics.

However, it is crucial to acknowledge that LAB viability undergoes a significant reduction during vinegar fermentation. While vinegar contains LAB, none of the vinegar studied so far met the minimum viable amount required for probiotic labeling. This observation emphasizes the challenges faced in harnessing vinegar as a viable probiotic product.

To fully unlock the potential of vinegar as a probiotic, further studies are imperative. These investigations should focus on enhancing LAB viability during vinegar fermentation, identifying strains with enhanced acid resistance, and establishing appropriate dosage and consumption guidelines to ensure functional probiotic benefits. Meanwhile, studies are showing the benefits of ingestion of numerous vinegar types spread around the world, thus fitting them into the term postbiotic. Even so, more *in vitro*

and clinical studies must be conducted to support this hypothesis. The identification and characterization of specific LAB strains from vinegar that possess desirable probiotic attributes, such as acid resistance and potential health benefits, warrant extensive research to achieve a closely probiotic product. This knowledge will contribute to the development of innovative approaches in the food industry, leading to the production of functional vinegar-based products enriched with beneficial LAB strains.

CHAPTER TWO – A REVIEW ON ENZYME-PRODUCING LACTOBACILLI ASSOCIATED WITH THE HUMAN DIGESTIVE PROCESS: FROM METABOLISM TO APPLICATION

Manuscript published in *Enzyme and Microbial Technology* journal. DOI: https://doi.org/10.1016/j.enzmictec.2021.109836

Abstract

Complex carbohydrates, proteins, and other food components require a longer digestion process to be absorbed by the lining of the alimentary canal. In addition to the enzymes of the gastrointestinal tract, gut microbiota, comprising a large range of bacteria and

fungi, has complementary action on the production of digestive enzymes. Within this universe of "hidden soldiers", lactobacilli are extensively studied because of their ability to produce lactase, proteases, peptidases, fructanases, amylases, bile salt hydrolases, phytases, and esterases. The administration of living lactobacilli cells has been shown to increase nutrient digestibility. However, it is still little known how these microbial-derived enzymes act in the human body. Enzyme secretion may be affected by variations in temperature, pH, and other extreme conditions faced by the bacterial cells in the human body. Besides, lactobacilli administration cannot itself be considered the only factor interfering with enzyme secretion, human diet (microbial substrate) being determinant in their metabolism. This review highlights the potential of lactobacilli to release functional enzymes associated with the digestive process and how this complex metabolism can be explored to contribute to the human diet. Enzymatic activity of lactobacilli is exerted in a strain-dependent manner, i.e., within the same lactobacilli species, there are different enzyme contents, leading to a large variety of enzymatic activities. Thus, we report current methods to select the most promising lactobacilli strains as sources of bioactive enzymes. Finally, a patent landscape and commercial products are described to provide the state of art of the transfer of knowledge from the scientific sphere to the industrial application.

Keywords: Lactobacilli; Digestive enzymes; Probiotic products; Fructanases; Amylases

Introduction

Enzymes are part of different biological processes of importance to human health. In the gastrointestinal tract (GIT), the digestive enzymes contained in the lysosomes promote the digestion of the most varied substances taken from outside of the cell. These enzymes act in a coordinated manner transforming carbohydrates, proteins, and fats into their monomers for human cell absorption. Some examples of digestive enzymes include amylase and lactase produced in the salivary glands; pepsin(ogen) in the gastric glands; trypsin(ogen), pancreatic amylase, lipase, and nuclease in the pancreas; and maltase and lactase in the small intestine (Govindaraj et al., 2020; Welcome, 2018).

Enzymes produced by gut-colonizing bacteria have a complementary action in breaking down complex substances during human metabolism. This microbial consortium consists of various representatives of bacteria, archaea, viruses, and fungi revealing themselves as a highly complex ecosystem (Peng et al., 2021). It contains more than 10 12 bacterial colony-forming units per gram, where approximately 1000 species live in symbiosis with the host. Lactobacilli are part resident of the human GIT from its initial colonization (de Melo Pereira et al., 2018; Xanthopoulos et al., 2000). They are included in the metabolic-based lactic acid bacteria (LAB) classification, comprising, at this time, more than 260 valid species and 29 subspecies (/wwww.bacterio.net/genus/lactobacillus) (Raveschot et al., 2018). As their name implies, they can form more than 50 % lactic acid as the product of carbohydrate utilization (Claesson et al., 2007; Hugenholtz et al., 1998). Lactobacilli origin is believed to be of plant material; however, a combination of extensive loss and acquisitions of key gene via horizontal transfer played a major role in the evolution and adaptation of these organisms to different environmental niches, including fruits, vegetables, cereal grains, fermentation processes (milk, dairy products, and meat), and human and animal microbiota (oral, gastrointestinal, and genital tracts) (Cavanagh et al., 2015). Lactobacilli diverged from the *Bacillus* genus through the loss of genes particularly related to cofactor biosynthesis and sporulation (Makarova et al., 2006). On the other hand, the acquisition of genes of transporters for efficient carbon and nitrogen utilization explains the adaptation of this microbial group to nutritionally rich environments (Barrangou et al., 2003; Klaenhammer et al., 2005).

Since the first classification by Orla-Jensen in 1919 (Bernardeau et al., 2006; Salvetti et al., 2012; Zheng et al., 2020), the genus of *Lactobacillus* has been the subject to taxonomic variations due to the popularization of phylogenetic studies using molecular data and powerful computer analyses. Advances in molecular techniques (DNA-DNA hybridization, mol % G+C content, and rRNA gene sequencing) allowed greater knowledge about the complex taxonomy and phylogeny of the group (Claesson et al., 2008; Zheng et al., 2020). Recently, a new taxonomic reclassification revision of the genus was proposed by Zheng et al., (2020). This study evaluated whole-genome sequences based on the polyphasic approach, considering the average nucleotide identity (ANI), average amino acid identity (AAI), core-gene average amino acid identity (cAAI), core-genome phylogeny, signature genes, and metabolic or ecologic criteria. Thus, the *Lactobacillus* genus was reclassified into 25 genera, which are being adopted in new scientific publications (International Scientific Association for Probiotics and Prebiotics, 2020).

Studies have shown that several lactobacilli species can produce enzymes associated with the human digestive process (García-Cano et al., 2020). It has been found that lactobacilli administration complements the digestion of the host, breaks down complex food components, and releases bioactive molecules, such as short-chain fatty acids (SCFA), prebiotic polysaccharides, galactooligosaccharides (GalOS), conjugated linoleic acids, phenols, and bioactive peptides. However, the exact microbial mechanism of action during the human digestive process is poorly known and needs further studies, with *in vivo* studies being generally scarce. This review provides a comprehensive overview on digestive-associated enzymes produced by *Lactobacillus* species, selection methods of enzyme-producing microorganisms, and a patent landscape on emerging products.

Lactobacilli-derived enzymes

Different species of *Lactobacillus* and recent reclassified genera have been studied regarding the release of functional enzymes (Figure 1). They are generally isolated from diverse sources, including crops, fermented foods, intestines, and feces of humans and animals (Table 1). The enzymes covered and detailed in this review include lactase, proteases, peptidases, fructanases, amylases, bile salt hydrolases, phytases, and esterases.

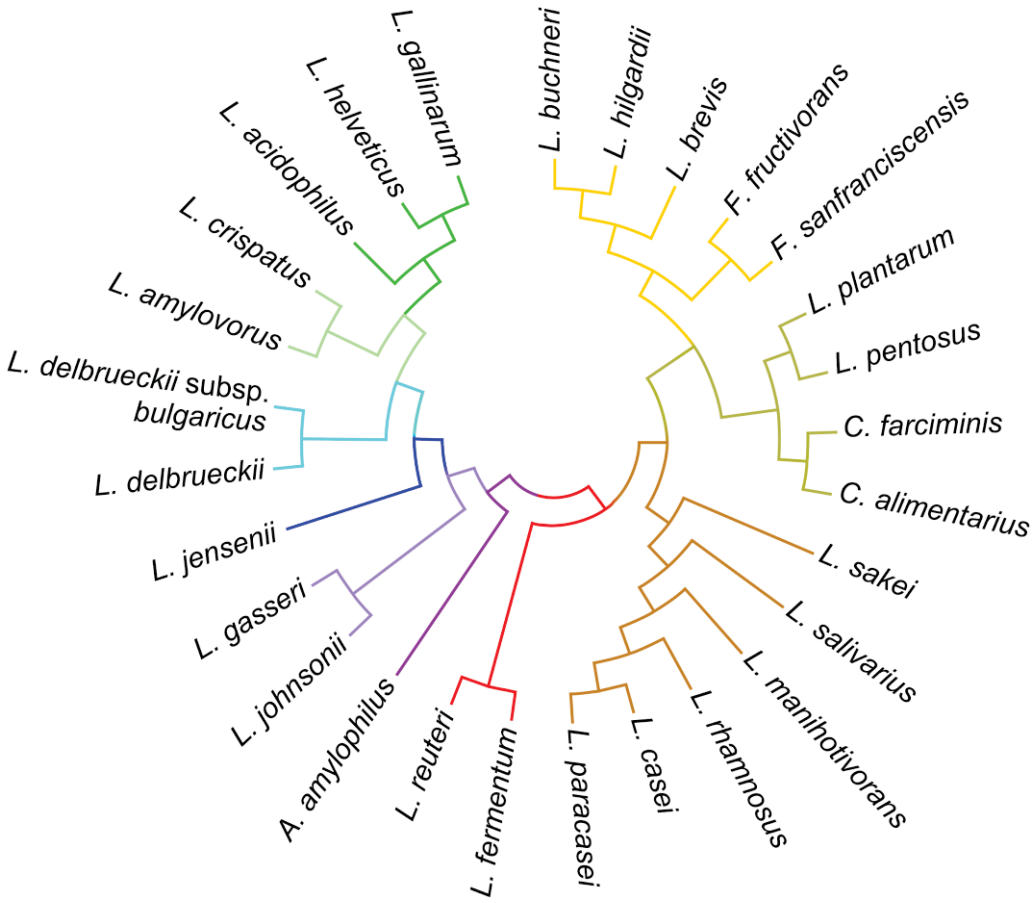


Figure 1. Maximum-likelihood tree based on 16S rRNA gene sequences showing the phylogenetic relationships of enzyme-producing lactobacilli associated with the human digestive process. The 16S rRNA gene sequences were retrieved from the GenBank database according to Zheng [15] and aligned with ClustalW. The phylogenetic tree was constructed using MEGA X version 10.1 program [192]. The type sequences used were: *Lactobacillus acidophilus* BCRC 10695 (access number: AY773947.1), *Lactobacillus johnsonii* ATCC 33200 (access number: AJ002515.1), *Limosilactobacillus fermentum* CIP JN175331.1), 102980 (access *Lactiplantibacillus* number: *plantarum* NRRL B-14768 (access number: AJ965482.1), *Limosilactobacillus reuteri* (access number: MN865144.1), *Lentilactobacillus buchneri* JCM 1115 (access number: AB205055.1), *Ligilactobacillus salivarius* ATCC 11741 (access number: AF089108.2), *Lacticaseibacillus casei* ATCC 393 (access number: AF469172.1), *Lactobacillus helveticus* DSM 20075 (access number: AM113779.1), *Lactobacillus gallinarum* ATCC 33199 (access number: AJ417737.1), *Lactobacillus amylovorus* DSM 20531 (access number: AY944408.1), *Companilactobacillus farciminis* ATCC 29644 (access number: M58817.2), *Companilactobacillus alimentarius* DSM 20249 (access number: M58804.2), *Lentilactobacillus hilgardii* DSM 20176 (access number: M58821.2), *Fructilactobacillus fructivorans* KCTC 3543 (access number: NR_036789.1), *Fructilactobacillus sanfranciscensis* ATCC 27651 (access number: X76327.1), *Levilactobacillus brevis* ATCC 14869 (access number: M58810.1), *Lactiplantibacillus pentosus* JCM 1558 (access number: D79211.1), *Lacticaseibacillus rhamnosus* JCM 1136 (access number: D16552.1), *Lactobacillus delbrueckii* BCRC 12195 (access number: AY773949.1), *Lacticaseibacillus paracasei* DSM 5622 (access number: D79212.1), *Lactobacillus delbrueckii subsp. bulgaricus* ATCC 11842 (access number: NR_075019.1), *Lactobacillus crispatus* ATCC 33820 (access number: AF257097.1), *Latilactobacillus sakei* DSM 20017 (access number: AM113784.1), *Lacticaseibacillus manihotivorans* OND 32 (access number: AF000162.1), *Amylolactobacillus amylophilus* DSM 20533 (access number: M58806.2), *Lactobacillus gasseri* ATCC 33323 (access number: AF519171.1), and *Lactobacillus jensenii* ATCC 25258 (access number: AF243176.1).

Table 1. Enzyme-producing lactobacilli strains.

Microorganism	Matrix origin	Molecular weight (kDa)	Temperature optimum (°C)	pH optimum	Reference
Amylase					
L. acidophilus LEM 220	Chicken crop	-	55	5.5	(Wang et al., 2012)
L. acidophilus LEM 202	Chicken crop	-	55	5.0	(Wang et al., 2012)
L. acidophilus LEM 207	Chicken crop	-	40	6.4	(Wang et al., 2012)
L.plantarum (A6)*	Cassava roots	-	55	5.0	(Jiang et al., 2010)
L. fermentum Ogi E1*	Fermented maize doughs	-	45	5.0	(Haros et al., 2008)
*L. manihotivorans**	-	135	37	6.5	(Sreeramulu et al., 1996)
L. amylophilus GV6*	Starch industry waste	90	55	5.5	(Raghavendra and Halami, 2009)
L. paracasei B41*	Beverage boza, prepared from wheat	67	45	5.0	(Shoaib et al., 2016)
Bile salt hydrolase					
L. acidophilus NCFM	Human	-	-	-	(Masuda, 1981)
L. acidophilus O16 and *L. acidophilus* L1	Human intestine	126	-	-	(Ren et al., 2011)
L. acidophilus ATCC 43121	Porcine intestine	126	-	-	(Ren et al., 2011)
L. johnsonii 100	Human	-	-	-	(Kim et al., 2004)
L. fermentum KC5b*	Healthy human feces	-	-	-	(Franz et al., 2001)
L. plantarum NDVR*	Strain bank	-	-	-	(Franz et al., 2001)
L. johnsonii PF01	Piglet feces	37	70	5.5	(Patel et al., 2010)
L. fermentum MTCC 8711*	Fermented milk products	36.5	-	7.5	(Foley et al., 2021)
L. plantarum 80*	Human intestine	-	30 - 45	4.7 - 5.5	(Van Eldere et al., 1996)
L. plantarum 80 (ML80)*	Silage	-	-	-	(Reale et al., 2004)
L. plantarum WCFS1*	Human small intestine	38 - 36	-	-	(Joyce et al., 2015)
L. plantarum ST-III*	Chinese pickles	37, 39, 37, and 36	-	-	(Jones et al., 2012)
L. plantarum CGMCC 8198*	Silage	37.53, 36.14, and 35.65	-	-	(Li and Chiang, 2014)
*L. reuteri**	Pig feces	-	-	-	(Tamang et al., 2009)
L. buchneri ATCC 4005*	Strain bank	-	-	-	(Pulido et al., 2007)
L. reuteri CRL 1098*	-	80	37 - 45	4.5 - 5.5	(Lavilla-Lerma et al., 2013)
*L. salivarius**	Chicken	37	41	5.4	(Zotta et al., 2007)

L. helveticus, L. fermentum[*] *and L. gallinarum*	Human intestine	-	-	6.0	(Hayek and Ibrahim, 2013)
Phytase					
L. casei DSM 20011[*] and *L. plantarum* W42[*]	Fermented milks and plant fermentations	-	50	5.5	(Iqbal et al., 2011)
L. amylovorus and *L. plantarum*[*]	Culture collections	-	40	4.4	(Sun et al., 2020)
L. farciminis[*], *L. alimentarius*[*], *L. acidophilus, L. plantarum*[*], *L. fermentum*[*], *L. hilgardii*[*], *L. fructivorans*[*], *L. sanfranciscensis*[*], *L. brevis*[*]	Sourdough	50	45	4.0	(Kahouli et al., 2013)
L.pentosus CECT 4023[*]	Sourdough	69	50	5.0	(Liu et al., 2016)
L. rhamnosus[*], and *L. amylovorus*	Chicken intestine	-	-	-	(Wang et al., 2008)
L. casei MF50[*], *L. fermentum* MF25[*], and *L. plantarum* MF79[*]	Ethiopian injera (African soft pancake)	-	-	-	(Srinivasan et al., 2007)
L. brevis[*], and *L. plantarum*[*]	Southern Italian sourdough	-	-	-	(Putranto et al., 2020)
L. brevis[*] and *L. plantarum*[*]	Fermented Himalayan vegetables	-	-	5.8 - 6.0	(Mustafa et al., 2020)
L. brevis[*]	Cheese	73 - 34	120 - 110	3.4 - 3.4	(Mroczyńska et al., 2013)
L. plantarum[*]	Carper berry	-	-	-	(Beganović et al., 2013)
L. plantarum[*] and *L. paracasei*[*]	Cheese	-	-	-	(Goh et al., 2007)
L. plantarum[*]	Italian Cornetto di Matera sourdough	-	-	-	(Bhathena et al., 2008)
L. acidophilus, L. plantarum[*], *L. reuteri*[*] and *L. rhamnosus*[*]	Sweet potato	-	-	5.5	(Fritsch et al., 2017)

L. plantarum MTCC 1325*	Cereal-legume fermentation	-	-	5.5	(Kin et al., 2009)
L. pentosus SJ65*	Fermented Uttapam batter	-	35	4.5	(Liu et al., 2016)
*L. fermentum NKN51**	Yak cheese	29.9	60	5.0	(Abeijón Mukdsi et al., 2012)
Lactase					
L.acidophilus	Fermented Ragi	50	-	7,0	(Suri et al., 2019)
L. acidophilus ATCC 4356	Culture collection	-	45	6.5	(Bhatia et al., 2015)
L. delbrueckii ssp. *bulgaricus* DSMZ 20081 and *L. acidophilus*	Fermented camel milk	-	-	6.0 – 7.0	(G. X. Liu et al., 2011)
L. helveticus DSM 20075	-	75 - 35	60 – 55	6.5	(Iqbal et al., 2010)
L. fermentum K4*	Chinese traditional dairy products	72 - 35	45 – 50	7.0	(Neves et al., 2005)
L. delbrueckii subsp. bulgaricus DSM 20081	Bulgarian yogurt	105	45 - 60	7.5	(Carević et al., 2016)
L. crispatus ATCC 33820	American Type Culture Collection	-	45	6.5	(Di Stefano et al., 2007)
L. casei ATCC393*	Culture collection	-	-	-	(Silanikove et al., 2015)
L. pentosus KUB-ST10-1*	Soil of a dairy farm in Thailand	105	60 - 65	7.5 – 8.0	(Vasiljevic and Jelen, 2003)
L. plantarum WCFS1*	-	107	60	7.0 - 7.5	(Shoaf et al., 2006)
L. sakei Lb790*	Meat	110	55	6.5	(He et al., 2016)
L. reuteri L103*	Culture collection	-	-	6.0	(Indira et al., 2019)
Protease					
L. fermentum R6*	Harbin dry sausages	37.7	40	6.0	(Zhu et al., 2020)
L. paracasei TKU012*	Infant vomited milk	49	60	10.0	(Genay et al., 2009)
L. plantarum 1.13*	Bakasam (traditional fermented meat)	24.3	40	4.0	(Topisirovic et al., 2010)
L. plantarum PTCC 1896*	Breast fed infant	25	39	7.5	(Mtshali et al., 2010)
L. helveticus M92	-	-	-	-	(Wu et al., 2011)
Fructanase					
L. crispatus	Sourdough	-	-	-	(Chae et al., 2013)
L. reuteri 121*	-	-	-	5.0 - 5.5	(Chiang, 2013)

L. gasseri DSM 20604	Culture collection	83	-	-	(Begley et al., 2006)
L. gasseri	-	84	50	4.5 - 6.0	(Begley et al., 2006)
L. paracasei 1195*	-	139	-	-	(Kumar et al., 2013)
L. reuteri 121*	-	-	50	4.0 - 5.5	(Chiang, 2013)
L. crispatus DSM29598	Sourdough	-	-	-	(Jayashree et al., 2014)
L. jensenii	-	65	45	6.0	(Long et al., 2017)
L. reuteri TMW1.106*	Sourdough	-	-	-	(McAuliffe et al., 2005)
Esterases					
L. fermentum NRRL B-1932*	NRRL culture collection	27.1	37	6.5	(Molska and Reguła, 2019)
L. fermentum 11976*	Culture collection	-	-	-	(Bhathena et al., 2008)
*L. gasseri, L. acidophilus, L. plantarum** and *L. fermentum**	German Collection of Microorganisms and Cell Cultures	27 – 29	20 - 30	7.0 - 8.0	(Fritsch et al., 2017)
L. fermentum CRL1446*	Goat milk cheese	-	-	-	(Abeijón Mukdsi et al., 2012)

Note: *According to the new classification proposed by [8] species names have been changed to: *Limosilactobacillus fermentum* (formerly *Lactobacillus fermentum)*, *Lactiplantibacillus plantarum* (formerly *Lactobacillus plantarum*), *Limosilactobacillus reuteri* (formerly *Lactobacillus reuteri*), *Lentilactobacillus buchneri* (formerly *Lactobacillus buchneri*), *Ligilactobacillus salivarius* (formerly *Lactobacillus salivarius*), *Lacticaseibacillus casei* (formerly *Lactobacillus casei*), *Companilactobacillus farciminis* (formerly *Lactobacillus farciminis*), *Companilactobacillus alimentarius* (formerly *Lactobacillus alimentarius*), *Lentilactobacillus hilgardii* (formerly *Lactobacillus hilgardii*), *Fructilactobacillus fructivorans* (formerly *Lactobacillus fructivorans*), *Fructilactobacillus sanfranciscensis* (formerly *Lactobacillus sanfranciscensis*), *Levilactobacillus brevis* (formerly *Lactobacillus brevis*), *Lactiplantibacillus pentosus* (formerly *Lactobacillus pentosus*), *Lacticaseibacillus rhamnosus* (formerly *Lactobacillus rhamnosus*), *Lacticaseibacillus paracasei* (formerly *Lactobacillus paracasei*), *Latilactobacillus sakei* (formerly *Lactobacillus sakei*), *Lacticaseibacillus manihotivorans* (formerly *Lactobacillus manihotivorans*), and *Amylolactobacillus amylophilus* (formerly *Lactobacillus amylophilus*).

Lactase

Lactose is the main carbohydrate in milk and, therefore, the primary source of energy for all human being newborns (Lawrence, 1994). When ingested, lactose is hydrolyzed by lactase, called lactose-galactose hydrolase, a border membrane-bound enzyme. Then, glucose and galactose are absorbed by the intestinal cells and transported into the bloodstream (Vonk et al., 2012).

Lactose and its hydrolysis derivatives are essential to human development. It facilitates calcium absorption and supports a healthy protective gut microbiota against pathogens, increasing defense for infections and adequate feces consistency. Also, galactose is the primary source of the white matter of the growing brain (do Nascimento and Issler, 2003). In addition, it is believed that lactose might act as a primer for microbiota colonization of the intestine in the first period of life. After its establishment, colonic microbiota also contributes to lactose degradation, as reminiscent non hydrolyzed lactose in the small intestine passes into the colon to ferment (Vonk et al., 2012).

Lactobacilli have a vital role in the initial colonization of the GIT. Vaginal-delivered infants acquire high amounts of lactobacilli from their mothers at delivery, as well as during breastfeeding (Wall et al., 2009). Lactose is a readily fermentable carbohydrate used as the primary source of energy for LAB, through the β-galactosidase activity, which is reported in innumerous lactobacilli species (*L. acidophilus, L. helveticus,* and *L. johnsonii*), *Loigolactobacillus coryniformis* (formerly *Lactobacillus coryniformis*), *Lactiplantibacillus plantarum* (formerly *Lactobacillus plantarum*), *Limosilactobacillus reuteri* (formerly *Lactobacillus reuteri*), and *Latilactobacillus sakei* (formerly *Lactobacillus sakei*) (Akolkar et al., 2005; Ibrahim, 2018; Jimeno et al., 1984; Kim and Rajagopal, 2000; Kittibunchakul et al., 2019; B. Liu et al., 2011; Maischberger et al., 2010; Nguyen et al., 2007, 2012; Vasiljevic and Jelen, 2003). *L. delbrueckii ssp. bulgaricus* and *L. acidophilus* were reported for their high enzyme production combined with high specific activity (Ibrahim, 2018). During a lifetime, the dependency on bacterial degradation fraction of lactose increases as the activity of human lactase is age-dependent, being high in the first year of age and declines until adulthood is reached (Suri et al., 2019).

β-galactosidases

The β-galactosidases belong to 4 different glycoside hydrolase families (GH1, GH2, GH35, and GH42), and have been characterized in all life domains (G. X. Liu et al., 2011). Microbial sources are preferable due to their ease of fermentative production, high activities, and good stability (Iqbal et al., 2010). Lactobacilli encode β-galactosidases belonging to families GH2 and GH42, being GH2 predominant. All belong to the LacLM type which are encoded by lacL and lacM genes and have approximately 105 kDa (Nguyen et al., 2012).

LAB uses lactose permease to transport lactose into the bacterial cell, where the enzyme catalyzes the hydrolysis of the β-1,4-Dglycosidic linkage of lactose intracellularly, resulting in glucose and galactose. Glucose is, then, metabolized into lactic acid (Neves et al., 2005). β-galactosidases and the compounds released from their metabolism have exceptional physicochemical and physiological characteristics and are widely used in the dairy industry for lactose hydrolysis in milk and whey, for example, resulting in the facilitation of digestion, especially for lactose intolerant consumers (Carević et al., 2016).

Lactose intolerance is a condition in which a person cannot digest or absorb lactose due to a decrease in intestinal galactosidase (lactase) (Suri et al., 2019). When accumulated, lactose causes osmotic pressure resulting in diarrhea, bloating, abdominal pain, and flatulence (Di Stefano et al., 2007). It is estimated that about 75% of the world's adult population suffers from the condition to some degree (Silanikove et al., 2015). Some LAB species lessen lactose intolerance through their enzyme β-galactosidase (Indira et al., 2019). Studies have been reporting on the use of lactobacilli to alleviate lactose intolerance side effects (Akolkar et al., 2005). Some examples include *Lactiplantibacillus plantarum* (formerly *Lactobacillus plantarum*), *Lactobacillus bulgaricus, Limosilactobacillus reuteri* (formerly *Lactobacillus reuteri*), *Lactobacillus acidophilus*, and *Lacticaseibacillus rhamnosus* (formerly *Lactobacillus rhamnosus*) (Suri et al., 2019). Furthermore, microbial lactose metabolism releases sugar molecules easily absorbed by the human intestinal cells (Minj et al., 2020), including bioactive oligosaccharides (Figure 2). Depending on the source of β-galactosidases and amount of lactose, this enzyme catalyzes transglycosylation reactions, where galactose moiety is transferred to alcohol or some aromatic glucoside, leading to the production of different galactosides (Carević et al., 2016). The more lactose, the greater production of these galactosides (Liu et al., 2011).

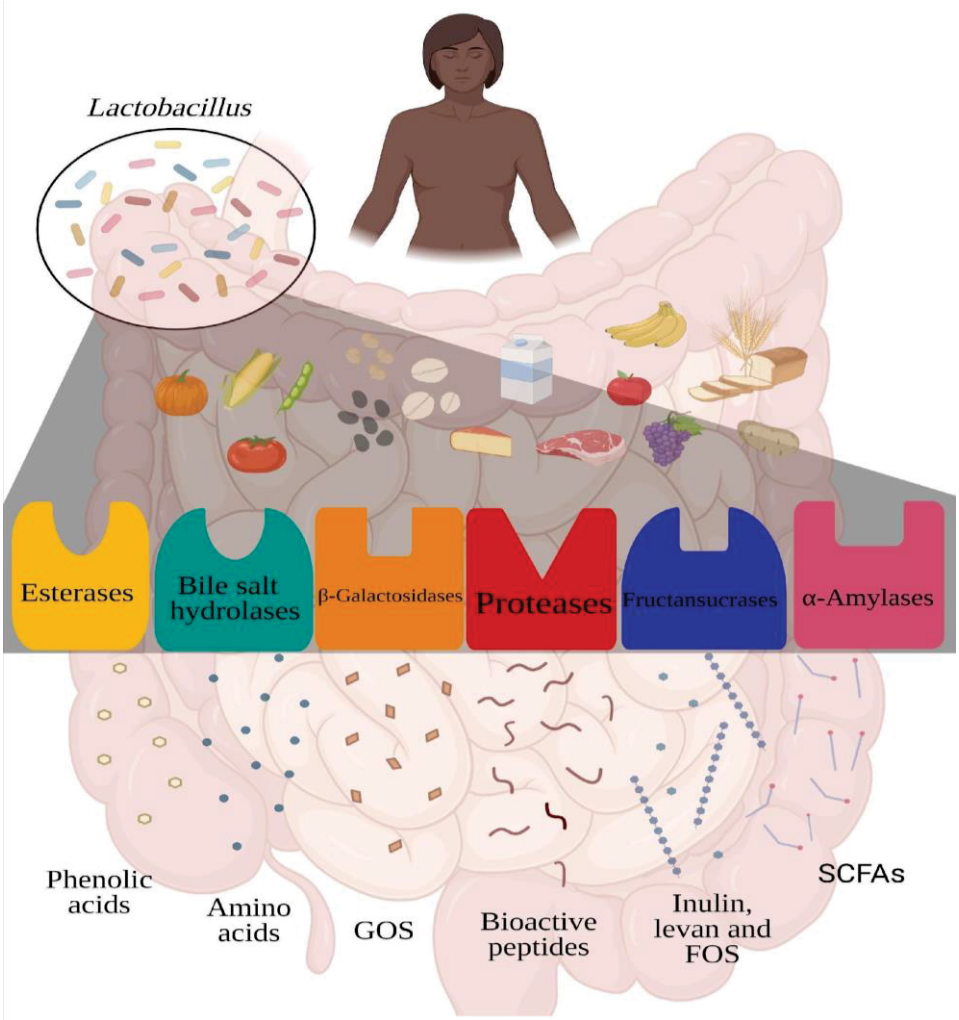


Figure 2. Bioactive compounds released by enzyme-producing lactobacilli on GIT (Created with BioRender.com).

Galacto-oligosaccharides (GalOS) are complex mixtures of nondigestible oligosaccharides known as prebiotic sugars (Nguyen et al., 2012). GalOS are one of the few prebiotics that meets the three criteria of (i) gastric acidity resistance; (ii) intestinal microflora fermentation; and (iii) growth and/or activity of intestinal bacteria associated with health and wellbeing (Maischberger et al., 2010).

GalOS enhances mineral absorption, increases beneficial bacteria population, decreases pathogenic bacteria, and improves immune response. Maawia et al., (2016) reported the improvement of Ca^{+2} and Fe^{+3}, Mg^{+2}, absorption on rats after three weeks of administration of GalOS produced through transgalactosylation of lactose catalyzed by β-gal from *Latilactobacillus sakei* Lb790 (formerly *Lactobacillus sakei* Lb790). GalOS ingestion also increases the number of desirable bacteria *in vivo* (Nguyen et al., 2007). Purified GalOS enhanced *Bifidobacterium* population on mouse gut microbiome (Monteagudo-Mera et al., 2016) and also on adults' fecal microbiota after three weeks of oral administration (Davis et al., 2010). In addition, GalOS purified from the Yakult product, which contains *L. sakei*, reduced the adherence of the enteropathogenic *Escherichia coli* on tissue culture cells (Shoaf et al., 2006). Positive effects on intestinal microbiota and immune response were also confirmed when GalOS was administered to elderly persons (Bhatia et al., 2015; Vulevic et al., 2008). However, GalOS are transiently formed as kinetic intermediates and are very complex mixtures consisting of numerous different oligosaccharides, then, depending on many factors to be studied in depth (Iqbal et al., 2010).

Proteases and peptidases

Proteases and peptidases (EC 3.4) are hydrolytic enzymes acting on peptide bonds, releasing peptides and amino acids from (poly)peptide chains. Endopeptidases or proteinases cleave preferably the internal portions of polypeptide chains, while exopeptidases act on the C- and N- terminals. They are also subdivided into acidic, neutral, and alkaline proteases, according to the pH of action.

Proteases and peptidases play an important role in the fermentation processes developed by LAB, especially in food products. The main function of the proteolytic system is the catabolism of protein molecules, oligopeptides, and amino acids for cell growth and maintenance, and this activity produces the desired "indirect" effects of flavor development, bitterness reduction, and release of bioactive peptides (Broadbent et al., 2011).In LAB, the proteolytic system is formed by a cell envelope-associated proteinase, specific transport systems for peptides and amino acids, and various cytoplasmic peptidases.

In addition to its vital role in protein catabolism, this system was identified as a mechanism of adaptation to high salt environments in *Lacticaseibacillus casei* (formerly *Lactobacillus casei*). Protease and peptidase activities enable cell homeostasis through the maintenance of adequate intracellular concentrations of amino acids, di- and tripeptides that act as osmoprotectants. Particularly, activities of the cell envelope-associated proteinase and a proline- type peptidase (X-prolyl-dipeptidyl aminopeptidase) increased in hypertonic media and, together with the PepI iminopeptidase, lost repression by peptides as observed at the transcriptional level. These enzymes were already purified from lactococci and lactobacilli (Piuri et al., 2003).

The cell-wall bound proteinase system was first characterized in *Lactococcus* strains, and the pioneer reports describing proteinases from lactobacilli date from the ’90's. Kojic et al., (1991) isolated and characterized a serine-type proteinase obtained from *L. casei* HN14 with activity toward β-casein. According to their assays, the proteinase gene was probably located in the chromosome, and not in the plasmid as occurs in lactococci (Kojic et al., 1991). The knowledge on lactobacilli proteases and peptidases has significantly evolved thanks to the development of genetic analysis tools like high-throughput genome sequencing and comparative genomic hybridization arrays. Liu et al. (Liu et al., 2010) performed a genomic analysis of the proteolytic system components, namely cell-wall bound proteinases, peptide transporters, and peptidases, of several genome sequences of lactic acid bacteria obtained from the National Center for Biotechnology Information (NCBI) microbial genome database, and the results obtained for lactobacilli strains are presented in Table 2.

Table 2. Distribution of cell-wall bound proteinases, peptide transporters, and peptidase genes in *lactobacilli* strains.

Strain	Proteinases	Peptide transporters			Peptidases					
		Opp	Dpp	Dtp	Aminopeptidases	Unique aminopeptidases	Endopeptidases	Dipeptidases	Tripeptidases	Proline peptidases
L. acidophilus NCFM	1	7	7	1	2	3	6	6	2	6
L. johnsonii NCC 533	2	8	0	1	2	3	7	7	2	4
L. gasseri ATCC 33323	0	8	0	1	2	3	7	6	2	5
L. delbrueckii subsp. *bulgaricus* ATCC 11842	1	6	10	0	2	3	4	4	2	6
L. delbrueckii subsp. *bulgaricus* ATCC BAA365	1	6	9	0	2	3	4	4	2	6
L. plantarum WCFS1	0	0	8	1	2	1	4	5	1	6
L. brevis ATCC 367	0	5	7	1	2	3	4	7	1	5
L. sakei 23 K	0	0	5	1	2	1	4	7	1	3
L. salivarius UCC118	0	0	5	1	2	1	3	3	1	3
L. casei ATCC 334	3	5	7	1	2	2	5	6	0	5
L. reuteri F275	0	0	0	1	2	1	2	7	1	4
L. helveticus DPC 4571	0	5	5	0	2	3	7	6	2	5

L. rhamnosus GG	3	5	6	1	2	3	6	5	1	6

Notes: Proteinases are represented by the cell-wall bound proteinases PrtP and PrtM. Peptide transporters are divided as "oligopeptides ABC transport system (Opp)" represented by OppA, OppB, OppC, OppD, and OppF; "di/tripeptides ABC transport system (Dpp)" represented by DppA/P, DppB, DppC, DppD, and DppF; and "di/tripeptides ion-linked transporter (Dtp)" represented by DtpT. Peptidases are represented by aminopeptidases PepC and PepN; unique aminopeptidases PepM, PepA, and Pcp; endopeptidases PepE/PepG, PepO, and PepF; dipeptidases PepD and PepV; tripeptidase PepT and proline peptidases PepX, PepI, PepR, PepL, PepQ, and PepP. Source: Adapted from [6

Recently, Qi et al., (2021) identified 16 peptidase genes in the genomic DNA of 50 sequenced strains of *Lactobacillus helveticus*, isolated from various environments including fermented dairy products, non-dairy products, and human feces. Aminopeptidases belonging to the superfamilies PepC and PepN and proline peptidases PepX were present in all genomes, usually with one gene, while proline peptidases PepL were absent in all strains (Qi et al., 2021). Zhong et al., (2021) evaluated the factors affecting proteolytic activity in *L. helveticus* and found that this feature was associated with acetate kinase (ackA) and two cysteine peptidases coding genes (pepC and srtA), while the distribution of cell envelope proteinases did not correlate (Zhong et al., 2021).

The beneficial effects of the presence of lactobacilli in the gastrointestinal tract can be in part attributed to the secretion of proteases and peptidases (Figure 2). As demonstrated by Caminero et al. (2019), the degradation of amylase trypsin inhibitors by lactobacilli reduced the inflammation caused by gluten in mice. Gluten is a mixture of glutamine- and proline-rich storage proteins, namely gliadin and glutenin, which are highly immunogenic and induce the adaptive immune response in individuals with celiac disease, wheat allergies, and associated disorders. Amylase-trypsin inhibitors, which are also present in gluten-containing cereals, have been shown to induce an innate immune response through the activation of the Toll-like receptor 4 (TLR4) in myeloid cells. In this sense, the administration of lactobacilli can be effective in reducing the inflammatory symptoms associated with wheat/gluten ingestion (Caminero et al., 2019).

Recently, Norouzbeigi et al., (2020) pointed out that probiotic strains can reduce the toxicity of gliadin toward intestinal cells, probably through the enzymatic hydrolysis of gliadin-derived toxic peptides. For example, the probiotic microorganisms used in sourdough fermentation promoted the degradation of gliadin resulting in a healthier product. The efficacy of lactobacilli, especially of *Companilactobacillus alimentarius* (formerly *Lactobacillus alimentarius*), *Levilactobacillus brevis* (formerly *Lactobacillus brevis*), *Lacticaseibacillus rhamnosus* (formerly *Lactobacillus rhamnosus*), *L. casei* (formerly *Lactobacillus casei*), and *L. paracasei* (formerly *Lactobacillus paracasei*), in reducing gliadin toxicity was also confirmed by recent studies. Besides, the administration of lactobacilli prevented the entrance of gluten-derived peptides into the cells. *Lact. rhamnosus* GG was effective in preventing the barrier dysfunction of the gut in the presence of gliadin, as demonstrated by *in vitro* (on Caco-2 cells) and *in vivo* (on Wistar rats) studies (Norouzbeigi et al., 2020).

The synthesis of prolyl endopeptidase (EC 3.4.21.26) by *L. acidophilus* 5e2 was optimized by Brzozowski and Lewandowska (Brzozowski and Lewandowska, 2014), to develop a product to hydrolyze prolamins, a group of storage proteins with high proline content present in cereals like wheat, barley, corn, and oat. Proline is a cyclic amino acid that prevents the enzymatic degradation of proteins. It occurs in biologically active peptides that act in the pathogenesis of depression, Parkinson's disease, and celiac disease (Brzozowski and Lewandowska, 2014). In this sense, they suggest that the administration of *L. acidophilus* could potentially prevent the development of these diseases.

Amylases

Starch has been part of the human diet since the dawn of civilization and is found in the form of semi-crystalline granules in legumes, cereal grains, roots, and tubers (Bertoft, 2017). This polysaccharide consists mostly of amylose and amylopectin. Amylose is a linear polymer composed of 1000–6000 glucose units with glycosidic bonds (α,1-4). Amylopectin is formed by a short linear chain (α,1-4) containing 10–60 glycoside residues and side chains (α ,1-6) composed of 15–45 glucose units (Maarel and Veen, 2002). When ingested, starch is partially hydrolyzed in the small intestine by α-amylases produced by the pancreas, but some factors, such as the proportion of amylose and amylopectin present in the starch molecule, the particle size, and cooking approach, can influence the digestion process (Maier et al., 2017). Starch molecules that are not digested in the small intestine are called resistant starch (RS) (Flint et al., 2012).

Studies have shown that RS can be fermented by bacterial communities that colonize the large intestine, resulting in the production of acids. This process tends to reduce the luminal pH, favoring populations of bacteria belonging to the phylum Firmicutes, mainly represented by LAB (Duncan et al., 2009). In addition, it is estimated that the metabolism of carbohydrates by colonic bacteria is responsible for the utilization of approximately 10 % of the calories that would be lost by the excretion of these polysaccharides through feces (Mcneil, 1984). The fermentation of starch by the gut microbiota also results in the production of SCFA, such as acetate, butyrate, and propionate (Figure 2).

The production of these compounds can generate several health benefits whereas (i) acetate can diffuse into the systemic circulation and be used in lipogenesis, (ii) butyrate is the main source of energy for the colon cells, and (iii) propionate is transported to the liver where it plays an important role in glycogenesis (Scott et al., 2008). Additionally, other effects, such as a reduction in the concentration of ammonia, phenol, and secondary bile acid (BA) (Nugent, 2005), may be associated with the prevention of inflammatory bowel disease and with colon cancer (Higgins and Brown, 2013).

An *in vitro* study conducted by Zampa et al., (2004) evaluated the effect of three polysaccharides (CrystaLean starch, xylooligosaccharides, and cornstarch) on the intestinal microbiota and SCFA production. For the fermentation of corn starch, the human fecal microflora was enriched with bifidobacteria and lactobacilli. Interestingly, the introduction of these two bacterial groups resulted in high production of butyrate (Zampa et al., 2004). Another study performed in rats showed that a high starch diet increased propionate production (Abell et al., 2011). This increase was associated with the bacterial groups *Parabacteroides distasonis, Ruminococcus Bromii*, and *Lactobacillus gasseri* (Abell et al., 2011).

It is known that, in general, starch hydrolysis is not common among LAB; however, some recent studies have reported amylolytic activity in some lactobacilli strains, being called amylolytic lactic acid bacteria (aLAB) (Table 1). According to Tou et al., (2006), the aLAB population present in fermented cereals accounts for only 12 % of the total LAB diversity. The sequencing of the complete genome of several *Lactobacillus* revealed the presence of genes for α-amylases in almost all strains, but a large part of these microorganisms does not express these enzymes due to mutations in the promoter, in the sequence encoding the signal peptide, or in the catalytic domain of the amylase (Petrova et al., 2013; Petrova and Petrov, 2012). Therefore, what differentiates aLAB from non-amylolytic LAB is their ability to produce several enzymes (e.g., exoamylases, endoamylases, debranching enzymes, and transferases) involved in the starch breakdown. Interestingly, several of these enzymes belong to the single family GH13 of glycosyl hydrolases, also known as α-amylases (Stam et al., 2006).

In silico analysis of the starch metabolism pathway by aLAB performed by Petrov, (2012) suggests that there are two possible directions for the catabolism of this polysaccharide to occur: hydrolysis to dextrin and then to glucose, or cleavage and conversion of the terminal glucose residues to α-D-glucose-1-phosphate. Therefore, these microorganisms may be essential for the maintenance of the microbial community of diverse starch-rich environments, since aLAB hydrolyze starch provides substrates for the growth of other non-amylolytic microorganisms (Haydersah et al., 2012; Merabti et al., 2019).

However, the probiotic properties of aLAB have been poorly explored, and the first papers characterizing the functional properties of these strains have been published recently (Freire et al., 2017; Gotcheva et al., 2018; Xu et al., 2020). *In vitro* tests were performed to evaluate the probiotic properties of 18 aLAB strains isolated from Brazilian indigenous beverages. *Lactiplantibacillus plantarum* CCMA 0743 (formerly *Lactobacillus plantarum* CCMA 0743) remained viable at acidic pH and in the presence of bile salts (Freire et al., 2017). Recently, Xu et al., (2020) also evaluated the probiotic and amylolytic properties of 132 LAB isolated from fermented cereal-based foods in China. Three strains (430, 445, and 472) were characterized as aLAB because they show high amylase activity (8.15, 9.23, and 8.06 U/mL in MRS-1% starch broth, respectively). These bacteria were identified as *L. plantarum* and showed antimicrobial activity, acid and bile salt tolerance, and aggregation capacity.

Although aLAB has shown promising results in *in vitro* tests, the ability to colonize the large intestine and produce SCFA has not been reported to date, and the impact of the administration of this bacterial group on the gut microbiota is yet to be explored. Additionally, another major technological advantage that aLAB presents is their ability to ferment starchy matrices without the need for starch pre-hydrolysis with exogenous enzymes (Espirito-Santo et al., 2014). This metabolic versatility has enabled the development of fermented starch-based foods with desired nutritional characteristics and texture, in addition to improving flavor and shelf life.

Fructanases

Fructans (inulin, levan, and fructooligosaccharides) are prebiotic polysaccharides ingested through several plant-based foods, such as vegetables, roots, tubers, fruits, and grains (barley, chickpea, lentil, lupin, rye, and wheat) (Dwivedi et al., 2014; Shoaib et al., 2016). These molecules are accumulated during plant development in vegetative stages (Veloso et al., 2017). When ingested, fructans cannot be digested by GI enzymes as mammals do not produce fructanases (Kazim, 2018). Instead, the fermentable carbohydrates fructans are utilized by the intestinal bacteria bifidobacteria, *Faecalibacterium prausnitzii*, and lactobacilli, enhancing their growth and producing SCFA that result in benefits for the host (Toledo et al., 2018).

Besides stimulation of indigenous probiotic bacteria, plant-derived fructans are known to improve mineral absorption, reduce gastrointestinal diseases, and enhance innate immune response with known anti- obesity, hypertension, oxidant, diabetes, and cancer actions (Charoenwongpaiboon et al., 2019; Ni et al., 2020). Fructans can also be synthesized in the gut from sucrose by lactobacilli enzymes fructansucrases and fructosyltransferases (FTF) (Anwar et al., 2010). Fructansucrase enzymes are reported in *Limosilactobacillus reuteri* (formerly *Lactobacillus reuteri*), *Lactobacillus delbrueckii,* and *Fructilactobacillus sanfranciscensis* (formerly *Lactobacillus sanfranciscensis*) (Loponen and Gänzle, 2018). FTF enzymes possess both levan or inulosucrase activity, polymerizing the fructose moiety of sucrose into either levan or inulin (Anwar et al., 2010). They have fructosyls by β-(2, 6) and β-(2, 1) glycosidic bonds, respectively, and a sucrose unit connects to the terminal (Ni et al., 2020). Inulosucrase is exclusively present in LAB (van Hijum et al., 2006) and has been reported in *L. reuteri, L. gasseri,* and *L. johnsonii* (Anwar et al., 2010).

The bacterial FTFs levansucrase and inulosucrase belong to the glycoside hydrolase (GH) 68 family. Glycosidic bond of sucrose is cleaved by hydrolysis to glucose and fructose; fructosyl residue is transferred to acceptor molecules and successive transfructosylation reactions form high molecular mass inulin and levan (Toledo et al., 2018).

Extracellular fructan sucrase is found in host-adapted lactobacilli, and even not being essential on their survival, enhance key adaptive advantages on biofilm formation and cell aggregation through the free exopolysaccharides released, being a key point on gut colonization (Li and Gänzle, 2020; Walter et al., 2008).

The resulting fructose-rich molecules are also speculated to be promising alternative sweeteners in the food industry because they are non-toxic, natural, have low-calorie, and are prebiotic (Toledo et al., 2018). However, its use is controversial.

Fructans are considered fermentable oligosaccharides, disaccharides, monosaccharides and polyols (FODMAPs), especially those on wheat and rye. Despite host beneficial effects already mentioned, when in high levels, fructans can result in functional gut disorders like irritable bowel syndrome (IBS) symptoms (Acín Albiac et al., 2020). IBS affects about 11% of the worldwide population and is frequently fructose malabsorbers (Gänzle, 2020). Fructan fermentation by ileal bacteria contributes to these side-effects as gases, H_2 as and CO_2, causing luminal distension, bloating, and osmotic diarrhea (Li and Gänzle, 2020). Low-FODMAP products are encouraged for intolerants, reduce fructan ingestion without avoiding wheat-containing products, and do not alter necessary daily fiber intake (Gänzle, 2020). Removal of fructans in cereal flours has been recently explored, and lactobacilli strains seem to be a promising tool. Studies show that lactobacilli extracellular fructanase genes (e.g., FruA) are not frequent and are acquired by lateral gene transfer. These polysaccharides can be efficiently metabolized by FruA-positive strains when other fermentable carbohydrate sources are scarce. Sourdough fermentation with *L. crispatus* DSM29598, for example, reduces more than 90% fructan content (Li et al., 2020). Extracellular fructanases were also characterized in *Lacticaseibacillus paracasei* (formerly *Lactobacillus paracasei*), *Lactobacillus crispatus* and *Lactobacillus amylovorans* (Loponen and Gänzle, 2018). However, to confirm product functionality, it is necessary for clinical trials with a large sample size and long-term treatments.

Bile salt hydrolases

Bile acid (BA) metabolism in the host's GIT is actively influenced by the gut microbiota through the action of microbial bile salt hydrolase (BSH). The production of bile starts in the host's liver, where cholesterol is converted into primary BA, cholic acid (CA), and chenodeoxycholic acid (CDCA), and is conjugated by N-acylamidates, mainly in taurine or glycine amino acids. These participate in the formation of micelles for the excretion of fat in the small intestine (Chiang, 2013). BA are digestive surfactants and have detergent properties, and, at high load, are lethal to numerous bacteria (Long et al., 2017).

Therefore, the ability to tolerate bile and BA is important for the survival of beneficial/probiotic bacteria in GIT, being a key point in the selection of probiotic strains according to the European Food Safety Authority (EFSA) criteria (de Melo Pereira et al., 2018).

The presence of BA in the GIT induces bacterial deconjugation by bile salt hydrolases. BSH catalyzes the deconjugation of bile salts by hydrolysis of the amide bond to release the glycine/taurine fraction from the steroid core (Begley et al., 2006). This function is recognized as the crucial bypass reaction in the BA biotransformation, regenerating free primary BA and CDCA, facilitating the microbial formation of secondary metabolites (e.g., deoxycholic BA (DCA) from CA, and lithocholic acid (LCA) and ursodeoxycholic acid (UDCA) from CDCA), as well as a range of intermediates (Long et al., 2017). This activity is well-documented for gram-positive intestinal bacteria of the genus *Lactobacillus*, especially in *L. acidophilus*, but also for *Limosilactobacillus fermentum* (formerly *Lactobacillus fermentum*), *Lactiplantibacillus plantarum* (formerly *Lactobacillus plantarum*), *Lactobacillus johnsonii*, *Ligilactobacillus salivarius* (formerly *Lactobacillus salivarius*), *Limosilactobacillus reuteri* (formerly *Lactobacillus reuteri)*, *Lacticaseibacillus casei* (formerly *Lactobacillus casei*), *Lentilactobacillus buchneri* (formerly *Lactobacillus buchneri*), *Lactobacillus gallinarum*, and *Lactobacillus helveticus* (Bateup et al., 1995; Chae et al., 2013; Christiaens et al., 1992; Corzo and Gilliland, 1999; Elkins et al., 2001; Gu et al., 2014; Jayashree et al., 2014; Lambert et al., 2008; McAuliffe et al., 2005; Pereira et al., 2003; Ren et al., 2011; Smet et al., 1994). Other genera were also reported for producing BHS, including *Bifidobacterium* (Kim et al., 2004), *Clostridium* (Masuda, 1981), and *Enterococcus* (Franz et al., 2001).

Lactobacillus strains from the human intestinal microbiota are capable of both glyco and tauro deconjugation of BA. Food-borne *Lactobacillus* are also capable of deconjugating; however, some prefer glycol deconjugation. Studies suggest that BSH genes are acquired horizontally in GIT and their activity is important at some level for lactobacilli to colonize the lower GIT (Elkins et al., 2001). BSH is highly substrate-specific for diverse types of bile (Patel et al., 2010), therefore, codifying multiple BSH genes confers greater fitness for this group, being a competitive feature for gut bacteria (Foley et al., 2021).

The intermediates and AA released by deconjugation can later be used as sources of carbon and nitrogen for bacteria survival (Van Eldere et al., 1996). This promotes increased population tolerance to bile salts and sensitivity to the host's defenses (Kociubinski et al., 2002). In addition, the process improves intestinal homeostasis, influencing the size and composition of BA and the intestinal microbiota (Joyce et al., 2015; Long et al., 2017). Available AA are also recognized as potent cell signaling molecules involved in the regulation of host glucose and energy metabolism, drug delivery metabolism, immune response modulation, and lipids and cholesterol levels, potentially promoting weight loss (Chiang, 2013; Joyce et al., 2015; Li and Chiang, 2014). Yogurt containing microencapsulated BSH-active *L. reuteri* taken twice per day for more than six weeks resulted in serum cholesterol lowering in hypercholesterolaemic adults (Jones et al., 2012). When deconjugated, BA are in a greater hydrophobic state compared to their conjugated molecules, thus, fewer are reabsorbed through intestine cells, resulting in increased excretion into the feces and a decrease in level of serum cholesterol (Choi et al., 2015). In addition to cholesterol lowering, some lactobacilli BSH activity were well correlated with *in vitro* anti-parasitic effects for the protozoa *Giardia duodenalis* (Allain et al., 2018; Travers et al., 2016). More studies need to be conducted to unravel this correlation.

Phytases

Phytate, also known as myo-inositol hexaphosphate (InsP6), is the principal storage form of phosphorus in plant-based foods (cereals and legumes) (Priyodip et al., 2017; Sharma et al., 2018). At the same time, phytate acts as an anti-nutrient compound because chelates ingest essential minerals (e.g., iron, calcium, magnesium, and zinc) and proteins in the human intestine (Saraniya and Jeevaratnam, 2015).

Humans are not able to produce phytases, however, it can be supplemented by ingestion of plants and microorganisms. The enzyme catalyzes the stepwise hydrolysis of IP6 to myo-inositol via penta- to monophosphates and free orthophosphates (Fischer et al., 2014). Bacteria possess more advantage for phytate utilization than plant-based enzymes due to substrate specificity and catalytic efficiency (Konietzny and Greiner, 2004). Probiotic bacteria from fermented foods showed high phytate degradation activity (Amritha and Venkateswaran, 2018).

Numerous studies have also reported lactobacilli species to be positively phytase (Saraniya and Jeevaratnam, 2015). As humans are unable to produce phytase and lack sufficient phytase-producing bacteria in their intestine (Bohn et al., 2008), phytase positive probiotic supplementation is well encouraged.

Phytase activity has a major role in cereal and vegetable food fermentation, being a desirable technological trait to be used in LAB starters culture selection (Palacios et al., 2005). Phytases dephosphorylate phytic acid, improving nutrient value of food, increasing substrate digestibility, and releasing organically bound phosphorus in the intestine (R. Sharma et al., 2018). Extracellular phytase increases the bioavailability of various chelated cations, such as iron, sodium, potassium, and calcium (Sharma and Shukla, 2020). Lactobacilli phytase activity is generally low compared to other bacterial genera such as *Bacillus* and *Escherichia* (Palacios et al., 2005). Even so, Sümengen et al., (2012) demonstrated that *Lactiplantibacillus plantarum* (formerly *Lactobacillus plantarum*), isolated from the traditional Turkish fermented drink shalgam, can produce a high amount of intra and extracellular phytase (984.50 U/mL and 494 U/g, respectively).

Phytase-positive, lactobacilli strains can also enhance antioxidant activity in the final product. The cause is still not clear; however, it might be due to the free phenolic content on substrates used for fermentation (e.g., isoflavone in soy milk). Antioxidant-rich foods have shown a highly beneficial effect on human health (Saraniya and Jeevaratnam, 2015). Despite advantages, most of the bacterial phytases are highly specific and their activity *in vivo* depends on the concentration of phytate, bacterial viability, optimum pH, the accessibility of phytate, presence of inorganic phosphate and other organic acids, phytase molecular mass, substrate specificity, and the presence of minerals (Fischer et al., 2014). Considering all these factors, further studies on safety and efficacy are necessary for phytases to be acceptable as a human food additive (Sharma et al., 2018).

Esterases

Ferulic acid (FA) is the principal hydroxycinnamic acid (HA) found in cereals, but also fruits and vegetables (Abeijón Mukdsi et al., 2012). The beneficial properties are due to its bioactive functions, including antioxidant, antimicrobial, and anti-inflammatory activities (Srinivasan et al., 2007). The colon is the major metabolic site to improve their bioavailability due to the action of feruloyl esterases (FE) by colonic microbiota (Russo et al., 2020).

Feruloyl esterases (FE) derived from microorganisms are highly desirable for the breakdown of ester bonds, releasing free FA form to be absorbed in the upper digestive tract of humans. FE has been characterized in *Lactobacillus amylovorus, Lactobacillus acidophilus, Companilactobacillus farciminis* (formerly *Lactobacillus farciminis*), *Limosilactobacillus fermentum* (formerly *Lactobacillus fermentum*), *Lactobacillus gasseri, Lactobacillus helveticus, Lactobacillus johnsonii* and *Lactiplantibacillus plantarum* (formerly *Lactobacillus plantarum*) (Esteban-Torres et al., 2013; Kin et al., 2009; Liu et al., 2016). Recently, Russo et al., (2020) administered *L. fermentum* on metabolic syndrome-induced mice fed with FA diet. Supplementation resulted in enhancement of physiological properties (decrease in body weight, adiposity index, blood leptin levels, fatty infiltration in hepatocytes, LDL-cholesterol, and triglyceride levels), improvement of inflammatory profile, and modulated intestinal microbiota with an increase in Bacteroidetes group.

Inhibition of harmful enzymes secreted by pathogenic organisms

It is well established that healthy intestinal biota is mainly composed of lactobacilli and *Bifidobacterium* (Mroczyńska and Libudzisz, 2010), while patients with pathologies in the GIT have higher counts of F*usobacterium, Bacteroides, Eubacterium, Proteobacteria*, and *Prevotella* (Kahouli et al., 2013; Mroczyńska et al., 2013). The high presence of specific fecal enzymes (β-glucosidase, β-glucuronidase, nitroreductase, and azoreductase) is considered a robust marker of dysbiosis in humans and animal models (Haberer et al., 2003; Mroczyńska et al., 2013). In general, these enzymes convert procarcinogens into potentially carcinogenic compounds, such as aromatic amines, transformed secondary bile salts, hydrogen sulfide, aglycones, acetaldehydes, and reactive oxygen species (Kahouli et al., 2013; Molska and Reguła, 2019).

β-glucosidase is mainly produced by *Bacteroides, Clostridium*, and *Enterococcus*; β-glucuronidase by *Escherichia, Staphylococcus, Clostridium, Bacteroides, Ruminococcus, Peptostrptococcus*, and *Eubacterium*; and nitroreductase and azoreductase by *Bacteroides, Clostridium, Enterococcus, Salmonella*, and *Staphylococcus* (Mroczyńska and Libudzisz, 2010).

Lactobacilli have a proven action in decreasing these fecal enzymes, mainly β-glucuronidase and β-glucosidase activities (De Preter et al., 2008; dos Reis et al., 2017; Haberer et al., 2003; Molska and Reguła, 2019; Zeng et al., 2019). The mechanisms of action include (i) exclusion of pathogenic bacteria through the secretion of antimicrobial substances; (ii) production of anti-inflammatory cytokines (interleukin 10); (iii) decrease of pro-inflammatory molecules, such as tumor necrosis factor and interleukin 8; and (iv) metabolization of dietary fiber into anti-cancer compounds, such as SCFA, conjugated linoleic acids, and phenols. Anticarcinogenic action can also occur through physical binding between the carcinogenic compounds and peptidoglycan present in the cell walls of some lactobacilli species (de Melo Pereira et al., 2018). *In vivo* studies demonstrate that these carcinogenic compounds can be eliminated together with the bacteria through feces (Burns and Rowland, 2004; dos Reis et al., 2017; Molska and Reguła, 2019).

Lactobacillus selection

Enzyme production is largely dependent on the *Lactobacillus* species or even strain at the genetic or physiological levels [149,150]. This highlights the need to carefully select the strains that will be used for enzyme production. The challenge is, therefore, to select highly efficient enzyme producers; that in conjunction meet the criteria for commercialization as probiotics established by the World Health Organization (WHO), including resistance to unfavorable conditions that the human body imposes, epithelium adhesion ability, antimicrobial activity, and safety assessment (de Melo Pereira et al., 2018). Fulfilling these criteria, the candidate probiotic is seen to be able to tolerate the stressful conditions of the human digestive system and exert functional properties. The methods and criteria for probiotic selection can be found in the review by de Melo Pereira et al., (2018).

Probiotic-producing enzymes can be evaluated by cultivating candidate strains in culture media supplemented with precursors (e.g., lactose, peptone-gelatin, starch, tauro-deoxycholic and glycol- deoxycholic acids, sodium phytate, inulin and ethyl ferulate, for the activity of lactase, protease, amylase, bile salt hydrolases, phytases, fructanases, and esterases, respectively) (Akolkar et al., 2005; Amritha and Venkateswaran, 2018; de Melo Pereira et al., 2018; Q. Li et al., 2020; Liu et al., 2016). Then, target-enzymes can be quantified mostly via spectrophotometry or fluorometric methods (Bairagi et al., 2002; Dipanjan Dutta, 2015; Suzer et al., 2008). Although phenotypic traits can be considered a "true" measure, it can have low reproducibility among laboratories. Thus, the use of Polymerase chain reaction (PCR) amplification with primers encoding target-enzyme genes can be considered (Bessler et al., 2009; Vázquez et al., 2017). The PCR products can be analyzed by gel electrophoresis or sequenced for confirmation of the amplified sequences. For β-galactosidase, for example, Kittibunchakul et al., (2019) used FwdNcoI, Rev1XhoI, and Rev2XhoI primers to amplify the gene lacLM from *L. helveticus*, whereas Nguyen et al., (2012) used F1, R1, and R2 to amplify lacZ gene from *L. delbrueckii subsp. bulgaricus*. Specific primers for other genes of enzymes produced by *Lactobacillus* are available in Table 3.

Table 3. List of specific primers for the selection of enzyme-producing lactobacilli.

Microorganisms	Enzyme	Primer	Sequence (5' – 3')	Ref.
L. helveticus DSM 20075	β-Galactosidase	FwdNcoI	GGCCCCATGGAAGCAAATATCAATTG	(Iqbal et al., 2010)
		Rev1XhoI	GGCCCTCGAGTGAATTTAGAATAAATGAAAATTC	
		Rev2XhoI	GGCCCTCGAGTTAATTTAGAATAAATGAAAATTC	
L. subsp. bulgaricus DSM 20081	β-Galactosidase	F1	GCTGCGTCTCCCATGAGCAATAAGTTAGTAAAAG	(Carević et al., 2016)
		R1	CGCGCTCGAGTTATTTTAGTAAAAGGGGCTG	
		R2	CGCGCTCGAGTTAGTGGTGGTGGTGGTGTTTTAGTAAAAGGGGC	
L. kefiranofaciens ZW3	β-Galactosidase	lacL F	GGATCCATGCAAGCAAATATTAAATGG	(Fritsch et al., 2017)
		lacL R	CTCGAGTTATTTGTGTAATCCATAATAGT	
		lacM F	GGATCCATGGATTACACAAATAAGCAATT	
		lacM R	CTCGAGTTAAAACTGGTTTAAGATGAAGG	
L. curieae M2011381	β-Galactosidase	s-1	CGCGGATCCATGGAAAATACAAAGA	(Zhu et al., 2020)
			CCGCTCGAGTTACGGCTCAATTTCA	
		s-2	CGCGGATCCATGACACCAGACATTA	
			CCGCTCGAGCTATTCTTTGTATTTTCCATAG	

		s-3	CGCGGATCCATGACACCAGACATTACATGGTTA	
			GTGCTCGAGCGGCTCAATTTCAAAAGCAAACTC	
L. fermentum K4	β-Galactosidase	Lf22b-F	GCACCATGGAAGCAGAGCTGAAATG	(G. X. Liu et al., 2011)
		Lf22b-R	TAGCTCGAGGTTAAGCTCGGGCAC	
		LfDuetL-F	GGTGGATCCTATGGAAGCAGAGCTGA	
		LfDuetL-R	GCGCTGCAGTTTGTGTAATCCATAGT	
		LfDuetM-F	GCTCATATGGATTACACAAATAAGCTG	
		LfDuetM-R	TTGAGATCTGTTAAGCTCGGGCAC	
L. helveticus CNRZ32	Proteinase	PrtH-for-1	GGTACTTCAATGGCTTCTCC	(Genay et al., 2009)
		PrtH-rev-1	GATGCGCCATCAATCTTCTT	
		PrtH-for-3	CTCAGCACCAGGTGGACATA	
		PrtH-for-2	CGATGATAATCCTAGCGAGC	
		PrtH-rev-2	TGGCAGAACCTGTGCCTA	
		PrtH2-for-1	CCAGCTAATAATCAAGACCA	
		PrtH2-rev-1	AACAGCATATTGAACTGCTC	
		PrtH2-for-2	GAAGACAAGGTGCTGGTCAA	
		PrtH2-rev-2	TAGCATTTTGGTCAAAGACA	
		PrtH2-for-3	GTTGGTGCCGCAACTAAATC	
L. helveticus	Proteinase	prtHf	CGGGAATCCTGATATGGAAACTTTAGGTAATC	(Broadbent

			ATTAAGACTAGCAAAGATTGGAAGATCTGAACTTG	et al., 2011)
		prtHr		
		prtH3f	GATGATCAAGCAGATGTAAAACCGGCAGAAG	
		prtH3r	ATTTACTGAAGAATTAGTCAAATGACCTGTTGTCGG	
		prtH2f	AAGCAAAGGATGTTGTTCCAAGTAAGCCA	
		prtH2r	CTCTCTTCCTTCTTACCAGTTGATGATTGAACT	
		prtH4f	CTGAAGCAGCAACTAATGATCCTGG	
		prtH4r	TGGATTAGGATCCGTTCTGGTTGTCAG	
L. plantarum BGSJ3–18	Proteinase	PrtP700	GCTTGAATTCGTTGTCGCTGCGGTTGT	(Topisirovic et al., 2010)
		PrtM700	GCATGAATTCAATGCACGATAAATGAG	
		P15C	AACCAAATCTGATGTTG	
		P06C	TTTCAGCGGAAGCAACT	
		PRTB10	GGTGTTGCTCCTGATGCCCAGC	
		PRTB20	CCCCGTTTAACAACTGCAAGTT	
		Jp23	GCTTGGATAGTAGCGTTAGC	
		Jp25	GGTGAACAAACTGAAGACG	
		prti2	CAACACCGGGACCACGGTG	
		IP6Xba	CTGATCGTGGACGGTGTTGC	
		U968	AACGCGAAGAACCTTA	

		L1401	GCGTGTGTACAAGACCC	
		pREV	TCGGGATTACCAAACATCA	
		paraF	GTCACAGGCATTACGAAAAC	
		pentF	CAGTGGCGCGGTTGATATC	
		plantF	CCGTTTATGCGGAACACCTA	
Lactobacillus sp.	Esterase	Est-1	GCTAATTTGTAACCGTATCCGCC	(Mtshali et al., 2010)
		Est-2	CGCGCATGTTAACTTTTAGTAGAAC	
L. casei	Esterase	estC	CACAATTTTGTCTTGTACGGTG	(Wu et al., 2011)
		estC	GTGGCGAATCCAATAATCTCTC	
L. fermentum NRRL B-1932	Feruloyl Esterase	FumPCR 517	AGCCATTTTTATCCGTCCCT	
		FumPCR 1531	GGGGCCTTACAGCTTTACCT	(Liu et al., 2016)
		Fumfae 661	CATGAAAGTGAGTTGATA*CCATGG*AA	
		Fumfae 1411	TC*GGATCC*CGGTTTAAGAAATC	
L. helveticus DSM 20075	Cinnamoyl Esterases	GASS-for	TATACCATGGTTATGAAGTTAAAGAAAAAG	(Fritsch et al., 2017)
		GASS-rev	TATATCTAGACGAAAAGTATTATTATCTTG	
L. acidophilus		REU-for	TATACCATGGAAATAACAATCAAACG	
		REU-rev	TATATCTAGACGATTTTTTAAAAAGTTAGCTAC	
L. gasseri DSM		PLA-for	TATACCATGGTTATGACATCGATGGAATTTAAG	

		PLA-rev	TATATCTAGAAATTTAAACGCGGCCAGTGCTAA	
L. plantarum WCSF1		FERM-for	TATACCATGGAAGTTGCAATCAAGA	
		FERM-rev	TATATCTAGATTGTTTAAGAAATCGGCCAC	
L. reuteri		HELV-for	TATACCATGGTTATGTCCCGCATTACGATTG	
		HELV-rev	TATATCTAGATTAAACGCAGGTTTTAAAAATTGC	
L. fermentum		ACID-for	TATACCATGGTTATGTCTCGCATTAACAATTG	
		ACID-rev	TATATCTAGACGAAATAGGGGCTTCAAAAATTC	
		BIF-for	TATACCATGGTTATGACGACGAGCACACATAC	
		BIF-rev	TATAGTCGACCGCCACCTCATGATGCGTC	
L. plantarum CGMCC No. 8198	Bile salt hydrolase	bsh1-F	GCCGGATCCGATGTGTACTGCCATAAC	(Li and Chiang, 2014)
		bsh1-R	CGCCTCGAGGTTAACTGCATAGTATTGTGC	
		bsh2-F	CGGCTGGATCCGATGTGCACTAGTCTAAC	
		bsh2-R	ATACTCGAGATGGGCCGCTGGCAAGGTG	
		bsh3-F	CGTGGATCCGATGTGTACTAGTTTAACGATTC	
		bsh3-R	TGTCTCGAGGTTTGCTAACCGGAACTGTTGG	
		bsh4-F	CTTGGATCCGATGTGTACCAGCTTAACTTATC	
		bsh4-R	ATACTCGAGATCGGCAGGGAAGAGGTACGGT	
		bsh1′-F	GGTATAAAAGCATTGTTATATCGGT	
		bsh1′-R	TTAAACCCAACTTACCATCGAGC	

L. fermentum NCDO394	Bile salt hydrolase	LfBsh1F	AATACATATGTGTACTGCCATAACT	(Kumar et al., 2013)
		LfBsh1R	AATACTCGAGTTAGTTAACTGCATAGTATTG	
L. salivarius	Bile salt hydrolase	bshF	CGCGGATCCATGTGTACAGCAATTACTTT	(Zotta et al., 2007)
		bshR	CGCTCGAGATTCAACTTATTTATTATTTGT	
L. acidophilus NCFM	Bile salt hydrolase	*bshA*	AAAGTCGACGAAAAGGGGCTTGGTA	(Masuda, 1981)
			AAGAATTCCCATCAGGTTGTTCTAC	
L. fermentum NKN51	Phytase	phyLf	AATCCATGG TGACCACCGTGTCTTTAAATCGCCATAAGCTTTTA	(Liu et al., 2016)
		phyLr	ATGATGATGATGATGATGAATGAGGTAGAGTTGTTTGAG	

As enzyme production is highly variable within the same species, it is important to evaluate the inter- or intraspecific diversity in a given lactobacilli collection. DNA fingerprinting approaches, including Random Amplified Polymorphic DNA (RAPD), Repetitive Element Palindromic PCR (rep-PCR), and Pulsed-field Gel Electrophoresis (PFGE), can be used to achieve strain-level identification. Whole genome sequencing (WGS) can also be applied as a more sophisticated strategy that, in addition to strain-level identification, provides knowledge about functional elements, gene function annotation, and comparative genome analysis. Finally, an *in vivo* assessment of the predicted enzyme activity must be performed to validate *in vitro* predictions (Lafarga et al., 2015).

Patents and commercial products

Over the past decades, the world has been continuously driven into an industrial-innovative economy, which is characterized by the transference of knowledge from the scientific sphere to the industrial application (Sanalieva et al., 2018). In this sense, patent databases can be considered a reliable source of measurement and evaluation of this technological innovation factor (Kessler and Sperling, 2016). According to a survey performed on the Patent Inspiration database (https://www.patentinspiration.com/), a total of 248 documents were deposited over the past 20 years associated with the production and application of lactobacilli-derived enzymes to human health benefits. The terms “Lactobacill* OR lactic acid bacteria AND enzym* OR protease OR phytase OR peptidase OR amylase OR lactase OR fructanase OR hydrolase” were used as keywords for search in title or abstract, and the Cooperative Patent Classification (CPC) codes A21 (baking, edible doughs); A23 (foods or foodstuffs), and A61 (medical or veterinary science; hygiene) were locked up to provide the desired results. From the initial search, only nine patent publications protected processes and products related to the proposed theme (Table 4). The heterogeneous distribution of applicants shows that this research field has a broad market application worldwide.

A published patent document proposes the ingestion of functional milk containing selected *L. acidophilus* and *L. bulgaricus* for the treatment of health disorders, such as IBS and peptic ulcer disease, associated with methane production in the small intestine (CH698855A2).

The synthesis of this volatile organic compound by methane-forming bacteria is associated with the ingestion of lactulose, a disaccharide commonly formed during milk ultra-high temperature treatment (Pimentel et al., 2003; Rasooly and Herold, 2011). Despite the document not providing the biochemical process involved for supporting such claims, a plausible reason for the health-promoting effect can be associated with the production of β-galactosidase produced by *Lactobacillus* species (Fara et al., 2020; Silvério et al., 2016).

The Food Science Institute from Tohoku University (Sendai, Japan) described the use of selected strains of *L. gasseri* and *Limosilactobacillus mucosae* (formerly *Lactobacillus mucosae*) for the amelioration of lactose intolerance due to high lactase production (JP20060175897). Although caplets of lactase are already commercially available and provide a complete alleviation of lactose intolerance symptoms, the effects are only observed during caplet intake. A study conducted by Almeida et al., (2012) revealed that the consumption of probiotic *Lactobacillus* species may provide long-term (three months) benefits, such as reduction of breath hydrogen concentration, pain, abdominal distention, and diarrhea, even after the probiotic uptake discontinuation. This result supports the constant search for new probiotic strains capable of producing lactase and colonizing the GIT for the alleviation of lactose intolerance, a condition that affects approximately 70 % of the adult world population (Ugidos-Rodríguez et al., 2018).

The coeliac disease is an immune-mediated enteropathy associated with the accumulation of proline- and glutamine-rich peptides present in grains (e.g., wheat, rye, and barley), which are resistant to gastric and pancreatic enzymes (Lindfors et al., 2019). The presence of these peptides in the lower gastrointestinal tract leads to the expression of autoantibodies that promote mucosal damage on the intestinal cells, thus affecting the absorption of nutrients and causing severe gastrointestinal symptoms (i. e., vomiting, diarrhea, weight loss). The only effectively known treatment for this condition is a permanent gluten-free diet. A recently granted patent from Danone (US10603342B2) discloses the oral administration of *L. casei* with peptidase activity against 33-mer, 20-mer, 13-mer, and 18-mer peptides, related to the coeliac disease immune-stimulatory effect (Camarca et al., 2009). The purification and crystallization of the enzyme revealed the presence of the elongation factor Tu, a versatile prokaryotic protein associated with translational elongation, refolding of denatured proteins, attachment to gastrointestinal cells, and degradation of n-terminal blocked proteins (Ong et al., 2020) (US1060334B2).

Similarly, a partnership between the Actial Pharmaceutica LDA and VSL Pharmaceutics Inc. suggested the oral administration of a LAB mix containing four lactobacilli (*L. acidophilus, L. plantarum, L. casei,* and *L. delbrueckii subsp. bulgaricus*), three *Bifidobacterium* species (*B. infantis, B. longum,* and *B. breve*), and *Streptococcus thermophillus* to promote the degradation of gliadins via proteolytic action from proline iminopeptidase, aminopeptidase, dipeptidase, prolinase, prolidase, dipeptidyl peptidase, tripeptidase, prolyl-endopeptidase, and endopeptidases (WO2006097415A1). In both cases, it is claimed that these lactobacilli strains can be used as a therapeutic treatment for celiac subjects or for those who have a high susceptibility to this condition.

According to recently patented technologies (Table 4), probiotic supplementation of enzyme-producing *Lactobacillus* is the main economical application, a market niche stimulated due to the numerous studies reporting the functional benefits of probiotic lactobacilli (de Melo Pereira et al., 2018; Garcia-Castillo et al., 2019; Mattia and Merker, 2008; Nami et al., 2014; Oh et al., 2018). The search of enzyme-producing strains was pioneered by the Chr. Hansen (Denmark) with the isolation and commercial application of the *L. acidophillus* DDS-1 in 1959, a high lactase-producing strain used for ameliorating the symptoms of lactose intolerance (Pakdaman et al., 2016). In 1990, Wakunaga of America (USA), a biotechnology industry recognized to produce food supplements, implemented the Kyo-Dophilus product line, containing the probiotic strains *L. gasseri* KS-13, *Bifidobacterium bifidum* G9-1, and *Bifidobacterium longum* MM-2. According to the manufacturer, probiotics can relieve digestive discomfort and aid in nutrient digestion, properties commonly associated with the production of lactase, amylase, proteases, and peptidases from *L. gasseri* strains (Azcarate-Peril et al., 2008; Lewanika et al., 2007; Saito, 2004). In 1995, Lallemand (Canada) developed a combinatory formula containing the high-lactase-producing strains *L. rhamnosus* Rosell®-11 and *L. helveticus* Rosell®-52, which were proven to reduce abdominal pain, nausea, bloating, and flatus from children with lactose malabsorption (Rampengan et al., 2010).

Table 4. Data obtained from the patent survey on Patent Inspiration database (https://www.patentinspiration.com/) regarding the application of enzyme-producing *lactobacilli* strains to human health benefits.

Patent Number (Publication)	Title	Resume	Country	Applicants	Granted	Citations	Year (Publication)
US10603342B2	Lactic acid bacteria for coeliac disease	Discloses the oral administration of the *L. rhamnosus* strain capable of producing elongation factor Tu enzyme that promotes the degradation of proline-rich peptides associated with coeliac disease	France	Gervais Danone SA	Yes	0	2020

KR102021883B1	*L. fermentum* Lm1016 and composition for preventing or treating inflammatory diseases and metabolic diseases comprising the same	The present invention relates to a *L. fermentum* LM1016 and composition for preventing or treating inflammatory or metabolic diseases through enhanced superoxide dismutase production	South Korea	Jeonnam Bioindustries Foundation	Yes	0	2020
WO2020157166A1	*L. mudanjiangensis* strains and cellulase derived therefrom	Selection *Lactobacilus* strain characterized by the expression of a novel cellulase enzyme, which has numerous applications in several fields	Belarus	Antwerpen University	No	0	2020
US9301983B2	Composition and method of *L. reuteri* GMNL-89 in treating type-2 diabetes	Use of a selected *L. reuteri* strain focused on the Diabetes Type-2 treatment through enhanced superoxide dismutase production and improvement of insulin utilization	Taiwan	Genmont Biotech Inc.	Yes	4	2016

US20140725 44A1	Polybacterial preparation with health benefits: antioxidant effect, reduction of cholesterol concentration, anti-inflammatory immunomodulating effect and release of bioactive peptides inhibiting angiotensin-converting enzyme	Claims the application of selected strains of *L. plantarum, L. helveticus,* and *L. gasseri* on the reduction of blood pressure through inhibition of angiotensin I and production of superoxide dismutase	Bulgaria	LB Bulgaricum PLC	No	1	2014
CH698855 A2	Food additive for human and animal consumption consists of a stable dispersion of lacto-bacteria cultures in milk	The invention relates to a probiotic food additive and pharmacologically effective medication comprising *L. acidophillus L. bulgaricus* for the reduction of methane formation in the lower gastrointestinal tract	Germany	Michael Heendorf	No	0	2009

WO2008001676A1	Lactic acid bacterium for amelioration of lactose intolerance	Discloses a method for selecting lactic acid bacteria belonging to the *Lactobacillus* genus with enhanced enteroadherence property and lactose-degrading enzyme	Japan	Tohoku University	No	6	2008
WO2007023896A1	Muscle pain suppressant and functional food for muscle pain suppression	A fermented milk product containing *L. helveticus* cells capable of producing antioxidant enzymes (*i.e.,* superoxide dismutase) for muscle pain suppression	Japan	Calpis Co.	No	0	2007

WO2006097415A1	Mixture of at least 6 species of lactic acid bacteria and/or bifidobacteria in the manufacture of sourdough	Proposition of a selective mix containing at least four *Lactobacillus* species capable of producing a vast range of peptidases and proteases that would be supplemented to coeliac persons to promote the degradation of gliadins	Portugal / USA	Actial Farmaceutica LDA (PT) and VSL Pharmaceusticals Inc. (USA)	No	17	2006	

During the same period, the market sales showed a fastidious increase due to the regulation via Dietary Supplement Health and Education Act (DSHEA), allowing the application of probiotics to be commercialized as dietary supplements or food substances without pre- marketing approval or rigorous requirements imposed on drugs or additives containing therapeutic or medicinal claims (Gogineni et al., 2013). Despite the favorable economic impulse, this regulation flexibility may lead to the use of unsafe or ineffective probiotics, since manufacturers are the ones responsible for collecting data about adverse events reported (Gogineni et al., 2013). The restriction imposed by the DSHEA redirects the vigilance authority to the Food and Drugs Administration, which has more stringent legislation for the commercialization of such products. This is one of the reasons for the existence of few probiotic products with lactobacilli strains containing therapeutic purposes in the current market.

Administration

To achieve the intended functional effects, probiotics or their enzymes orally administered must thrive through the stressful conditions imposed by the processing, storage, and passage through the GIT, such as temperature fluctuations from freeze-drying and spray- drying and acidic pH and bile salts exposure (Asgari et al., 2020; de Melo Pereira et al., 2018). In this sense, several strategies have been proposed to provide additional “barriers” for the correct disposal of cell suspension on the colon (10^7 -10^9 CFU per oral dose) (Vinderola et al., 2011). The most common protective formulation of probiotics is the addition or fermentation of dairy or non-dairy matrices, such as cheese, ice cream, cereals, and vegetables (Castro et al., 2015; Cruz et al., 2009; Leone et al., 2017). The eligibility of a given product to be a carrier is based on i) presence of the required nutrients for probiotic growth; ii) buffering effect; iii) content of sugar, protein and lipid that can confer a higher tolerance to the gastric enzyme degradation and bile salts; iv) anaerobic condition (preferably) (Flach et al., 2018). Besides food, encapsulation of probiotic microorganisms in biocompatible polymers (e.g., amylose, chitosan, alginate, and cellulose polymer) is an easy and fast method for the gradual release of the probiotic via enzymatic degradation, redox reaction, or pH oscillation (Asgari et al., 2020).

Due to the easy disruption by physicochemical actions, the subsequent coating of these matrices with polysaccharides or residual carboxylic groups allows the increased resilience of the microorganism to gastric enzymes and bile salts, the delivery of probiotics on the upper-GIT, and a more efficient colonization (Chávarri et al., 2010; Cook et al., 2011; Fávaro-Trindade and Grosso, 2002).

The on-growing knowledge of the therapeutic properties of probiotics provided by the omics technologies and genetic engineering allowed the emergence of a new category within this field: the next generation probiotics (NGP) or living biotherapeutic (O'Toole et al., 2017). According to the FDA, these products contain a living organism, which is applicable to prevention, treatment, or cure of a disease or condition; but it is not considered a vaccine (Food and Drug Administration, 2016). One of the strategies used for the formulation of these products is the development of target-specific molecules to the human cell based on strong coating association or mechanistic insight that allows the delivery of the intended molecule to promote the healing factor (O'Toole et al., 2017). A recent study evaluated the delivery of *L. rhamnosus* encapsulated with a thiolated hyaluronic acid hydrogel (THAH) for the amelioration of pathogen-induced enteritis (Xiao et al., 2020). The THAH carrier was developed and molded in such a way that, when the disulfide bonds of THATH were exposed to the H_2S produced by the enteric *Salmonella*, the hydrogel would release the probiotic load. The *vivo* assay showed that infected mice treated with the NGP reduced completely the *Salmonella* levels, restoring the gut homeostasis and flora. Alongside the target-oral administration, some studies have revealed that lactobacilli species are able to reduce the murine colitis, gastric necrotic lesions, and multiple erosions of the small intestine after subcutaneous administration (Laudanno et al., 2006; Sheil et al., 2004). This alternative administration path eliminates the stressful and harsh conditions faced by the probiotics when passing through GIT, reducing costs and providing the insurance of a correct disposal of cellular concentration. This research opens new avenues for on-target delivery systems and the usage of more effective administration pathways of these NGP.

Concluding remarks

This review outlined enzyme-producing lactobacilli strains with a focus on functional aspects. The species *Lactobacillus acidophilus, Lactiplantibacillus plantarum* (formerly *Lactobacillus plantarum*), *Lacticaseibacillus casei* (formerly *Lactobacillus casei*), *Lactobacillus delbrueckii subsp. bulgaricus* and *Lacticaseibacillus rhamnosus* (formerly *Lactobacillus rhamnosus*) are the most frequent producers of enzymes including lactase, proteases, peptidases, fructanases, amylases, bile salt hydrolases, phytases, and esterases. These enzymes complement human digestive metabolism in a two-way street, where host-adapted lactobacilli utilize complex, resistant, and non-degradable molecules as an energy source for GIT colonization. Then, digestive lactobacilli apparatus i) enhances food digestibility and nutrients bioavailability, ii) decreases malabsorption and intolerance side-effects, and iii) releases bioactive molecules with functional properties that cannot be accessed by human metabolism. However, the high complexity of host digestive metabolism pathways, variable conditions faced in the GTI (e.g., temperature, pH, diet content), and lack of detail on enzyme mechanisms are barriers to the approval of novel probiotic products. Nevertheless, *in vivo* studies using enzyme positive lactobacilli supplementation enhances mineral absorption and intestinal homeostasis, modulating intestinal microbiota, reducing systemic inflammation, and mitigating metabolic syndrome.

Lactobacillus is already part of the indigenous human microbiota and is widely accepted for safe consumption, being a plausible candidate for human supplementation. Other enzymes produced by *Lactobacillus* species, such as lipases, arabinases, glutaminases, and glycohydrolases, seek an in-depth assessment to establish their functional role. Identification of strains having multiple enzymatic actions and standardized *in vitro* protocols simulating stressful conditions of the human digestive system for selection of highly efficient enzymes is of utmost importance. This will facilitate *in vivo* studies and the approval of probiotic products by legislation, as well as avoid possible manufacturers' suspicious appeals.

CHAPTER THREE – PREDICTING THE MICROBIOME AND METABOLOME DYNAMICS OF NATURAL APPLE FERMENTATION TOWARDS THE DEVELOPMENT OF ENHANCED FUNCTIONAL VINEGAR

Manuscript published in *Fermentation* journal. DOI: https://doi.org/10.3390/fermentation10110552

Abstract

Natural vinegar fermentation is a complex process influenced by the interplay between microbial communities and metabolites. This study examined the interplay between the microbiome and the metabolome over a three-month period, with samples collected every ten days. Using Illumina sequencing and chromatographic techniques (HPLC and GC-MS), we mapped microbial shifts and metabolite profiles. Early fermentation showed a diverse microbial presence, including genera such as *Cronobacter*, *Luteibacter,* and *Saccharomyces*. A stable microbial ecosystem established between the days 15 and 70, characterized by the dominance of *Leuconostoc, Gluconobacter,* and *Saccharomyces,* which facilitated consistent substrate consumption and metabolite production, including various organic acids and ethanol. By day 70, Acetobacter prevalence increased significantly, correlating with a peak acetic acid production of 12.4 g/L. Correlation analyses revealed significant relationships between specific microbes and volatile organic compounds. This study highlights the crucial roles of these microbes in developing sensory profiles suited for industrial applications and proposes an optimal microbial consortium for enhancing vinegar quality. These data suggest that an optimal microbial consortium for vinegar fermentation should include *Saccharomyces* for efficient alcohol production, *Leuconostoc* for ester-mediated flavor complexity, and *Acetobacter* for robust acetic acid production. The presence of *Komagataeibacter* could further improve the sensory and functional qualities due to its role in producing bacterial cellulose.

Keywords: LAB; *Lactobacillus;* acetic acid; natural fermentation.

Introduction

Vinegar, with its rich historical background, holds significance as an acidic seasoning utilized in culinary practices and daily life, having served medicinally for centuries in treating wound healing, poison ivy, croup, stomachaches, high fevers, edema, infections, and ulcerations (Ho et al., 2017). Vinegar production involves two biochemical processes: alcoholic fermentation followed by acetic acid fermentation (AAF). In the first stage, sugars and/or starches are consumed by yeasts in anaerobic conditions, typically from the *Saccharomyces* genus (Pazuch et al., 2020). Subsequently, AAF of ethanol by acetic acid bacteria (AAB) occurs in an aerobic environment. The AAF microbial community comprises innumerous genera of AAB, such as *Acetobacter, Gluconobacter, Komagataeibacter*, and *Gluconacetobacter*. Nevertheless, species within the *Acetobacter* and *Komagataeibacter* genera notably demonstrate a robust capability for acetic acid production (Hata et al., 2023). In acetic fermentation, ethanol is converted to acetic acid through the action of two membrane-bound enzymes located on the outer surface of the cytoplasmic membrane (periplasmic side). Initially, alcohol dehydrogenase (ADH) oxidizes ethanol to acetaldehyde, which is further oxidized to acetic acid by aldehyde dehydrogenase (ALDH).

While all types of vinegar production follow the same sequence of biochemical steps, the composition of microbiota can vary significantly among them. Over the last twenty years, there has been significant progress in the field of studying fermented foods, attributable to advancements in next-generation sequencing, advanced mass analyzers, and other innovative tools (Shi et al., 2022). These technologies have substantially increased the capacity and sensitivity of research in the field. Particularly, culture-independent taxonomic methods, predominantly relying on high-throughput sequencing, have been frequently employed and revealed numerous previously undetected, non-dominant microbes. Various fermented foods, such as cheese, kefir, coffee, cacao, yogurt, and vinegar, have had their microbial genomes sequenced, highlighting the intricate roles and contributions of diverse microorganisms in the fermentation process (Siddiqui et al., 2023). In natural vinegar fermentation, NGS has been revealing non-dominant groups such as L*ichtheimia, Pediococcus, Xanthomonas, Kazachstania, Gardnerella, Streptomyces, Pantoea, Pseudomonas, Serratia, Prevotella,* and *Corynebacterium* (Kong et al., 2022; N. Li et al., 2023). The unique qualities of various vinegars around the world

are shaped by the specific raw materials used, the microbial environment, and the distinct brewing techniques employed (Gong et al., 2021).

The microbial composition of vinegar directly impacts on the product's quality and the formation of volatile compounds during fermentation. Diverse raw materials influence their physical and chemical properties, thus affecting the taste, aroma, and overall quality. Volatile compound profiles of vinegars are closely tied to the raw materials, their composition, and the production methods used (Gong et al., 2021). For instance, alcoholic fermentation is a crucial step because the alcohol produced serves as a precursor to many flavor compounds, such as ethyl acetate, isoamyl acetate, isoamyl alcohol, and benzaldehyde, significantly contributing to the final quality of vinegar (Jin et al., 2021; Wang et al., 2016). Conversely, acetic acid fermentation is responsible for oxidizing ethanol into acetic acid and is also essential for the development of flavor compounds including acetaldehyde, benzaldehyde, acetone, succinic acid, and diacetyl. Lactic acid bacteria are found in natural fermentation vinegars, occurring in minor proportions in some cases, while dominating the microbial community in others, depending on the specific fermentation matrix.

The application of controlled fermentations using mixed cultures—comprising lactic acid bacteria and yeast—can enhance the production process by yielding vinegars enriched with nutrients such as vitamin B, flavonoids, and amino acids. This approach not only ensures better microbial regulation but also promotes a targeted increase in the concentration of key volatile compounds, particularly ethyl lactate, ethyl caprate, and ethyl caproate (Li et al., 2023). Although lactic acid bacteria appear in lower proportions compared to the dominant acetic acid bacteria, they play a significant role in shaping the vinegar's sensory profile by contributing to the synthesis of esters and other compounds such as ethyl acetate, acetaldehyde, and diacetyl (Thierry, Anne Tomislav Pogačić, Magalie Weber, 2015).

Across European nations, a variety of time-honored vinegars, such as Italy's balsamic vinegar and Spain's sherry vinegar, are predominantly produced through liquid-state fermentation, primarily utilizing wine substrates (Haruta et al., 2006). In Asian countries, such as China, Japan, and Korea, vinegar production began around 1000 BC, and it is a highly appreciated ingredient commonly used to season dishes like seaweed salad, sushi, and boiled and steamed fish (Ho et al., 2017). In Brazil, vinegar consumption reaches 170 million liters per year, with 80% being ethanol vinegar. According to the

National Association of Vinegar Industries, Brazilian per capita consumption is 0.8 L, while in Europe and the USA, it reaches 1.8 L per capita (Pazuch et al., 2020). Globally, natural vinegar fermentation has been extensively studied; however, there is limited research on the process in Brazil. Brazil's diverse climate, in contrast to Asia and Europe, may lead to the development of new species and biochemical processes unique to the region.

This study investigated microbial dynamics and metabolite profiles during vinegar fermentation, addressing a notable research gap in Brazil's vinegar production history. Considering Brazil's distinct climate and traditional methods of vinegar fermentation, elucidating the microbial composition and metabolic pathways involved in this process can offer valuable insights into optimizing raw material selection, fermentation conditions, and processing techniques. These findings are crucial not only for enhancing vinegar production practices in Brazil but also for advancing the global understanding of vinegar fermentation processes.

Material and Methods

Fermentation and Sampling

An acetic acid inoculum (also known as "Mother of Vinegar") was collected from a private household that traditionally produces apple vinegar in Curitiba city, Paraná State, Brazil. The inoculum was maintained at ambient temperature (approximately 18.61 ± 2.72◦C) for 4 weeks prior to inoculation. Fermentation process was conducted traditionally in duplicate glass urns of 3 L total volume for 3 months. Fresh organic apples were purchased from a local city market and utilized at a rate of 0.5 kg per liter of must. The must consist of manually macerated apples and mineral water, while commercial white sugar was incorporated until achieving 20◦Bx. The alcoholic fermentation phase was conducted spontaneously, as traditionally practiced in Brazil. In the sixth week, acetic acid inoculum was added to start acetic fermentation in a proportion of 10%. The urns were kept at ambient temperature (approximately 18.61 ± 2.72◦C). Samples (20 mL) of fermenting vinegar were collected at intervals of 10 days (10 weeks total) to perform microbiological and metabolite target analyses. At each sampling point, the pH was measured using a digital pH meter (LUCA-210 model, Requipal, Curitiba, PR, Brazil).

DNA Extraction and Metataxonomic Analysis

DNA was extracted from each sample utilizing the DNeasy PowerSoil Pro Kit (Qiagen, Hilden, Germany), following the protocol provided by the manufacturer. Following extraction, DNA concentrations were measured using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The V3–V4 hypervariable regions of the bacterial 16S rRNA gene were amplified from the isolated DNA using primers 341F and 805R. For the amplification of the fungal ITS region, primers ITS 3S and 4R, tagged with Nextera indices, were used in accordance with the manufacturer's instructions (Illumina Inc., San Diego, CA, USA). Paired-end sequencing (2 × 250 bp) was performed on a MiSeq platform using the MiSeq v2 reagent kit (Illumina, San Diego, CA, USA). The resulting raw sequencing reads were analyzed using the QIIME (Quantitative Insights into Microbial Ecology) pipeline, where sequences shorter than 100 bp or containing more than one ambiguous base (N) were filtered out. High-quality sequences were aligned against the SILVA database using the UCLUST algorithm, and taxonomic classification along with the generation of operational taxonomic units (OTUs) was carried out at a 97% sequence identity threshold.

Bioinformatic Analyses

After completing the sequencing process, chimeric sequences were identified and removed, along with the reduction of noise during pre-clustering and taxonomic assignment, using the default settings of QIIME software version 1.9.0. Employing the UCLUST method (Edgar, 2010), sequences exhibiting greater than 97% similarity were categorized as identical operational taxonomic units (OTUs) according to the SILVA database and QUAST (Quality assessment tool for genome assemblies).

Co-Occurrence/Co-Exclusion Analysis

The relationships between variables were evaluated using Spearman correlation analysis, performed with R v4.2.3 and the corrplot package. Network diagrams were created and displayed with the open-source software Gephi v0.10.1, employing the Yifan Hu algorithm for node distribution. These maps, showing the Spearman correlation coefficients as edges, illustrate the complex interactions between microbial species and their impact on vinegar flavor profiles during fermentation. The relationships between variables were evaluated using Spearman correlation analysis, performed with R v4.2.3 and the corrplot package.

Consumption and Production of Substrates

The determination of sugar consumption and organic acid and ethanol production was performed through periodic sampling using high-performance liquid chromatography (HPLC) with slight modifications (de Oliveira Junqueira et al., 2019). A 2 mL aliquot was centrifuged at 6000× g for 15 min and subsequently filtered through a hydrophilic Polyethersulfone (PES) membrane with a pore size of 0.22 µm (Millipore Corp., Burlington, MA, USA). An aliquot of 100 µL of the filtered samples was injected into the HPLC system, which was equipped with an Aminex HPX 87 H column (300 × 7.8 mm; Bio-Rad, Richmond, CA, USA) and a refractive index (RI) detector (HPG1362A; Hewlett-Packard Company, Palo Alto, CA, USA). The elution of the column was conducted in isocratic mode using a mobile phase of 5 mM H_2SO_4 at 60 ◦C, with a flow rate set at 0.6 mL/min.

Secondary Metabolites Formation

The extraction of volatile compounds was performed using a headspace (HS) vial coupled to a SPME fiber (CAR/PDMS df75 µm partially crosslinked; Supelco., Saint Louis, MO, USA). For each determination, 2 mL of sample was stored in a 20 mL HS vial in triplicate. The SPME fiber was exposed for 30 min at 60 ◦C. The compounds were thermally desorbed into the GC injection system gas phase (GC-MS TQ Series 8040 and 2010 Plus GC-MS; Shimadzu, Tokyo, Japan) at 260 ◦C. The column oven temperature was maintained at 60 ◦C for 10 min, followed by two heating ramps of 4 and 10 ◦C/min until reaching the temperatures of 100 and 200 ◦C, respectively. The compounds were separated on a 95% PDMS/5% PHENYL column (30 m × 0.25 mm × 0.25 mm film thickness). The GC was equipped with an HP 5972 mass selective detector (Hewlett Packard, Palo Alto, CA, USA). The compounds were identified by comparison to the mass spectra from library databases (Nist'98 and Wiley7n).

Results and Discussion

Microbial Dynamics Profile During Spontaneous Vinegar Fermentation

A total of 2,044,774 paired-end reads were obtained from Illumina sequencing for prokaryotes and 2,148,806 for eukaryotes, grouped into 237 and 32 OTUs, respectively, at 97% sequence similarity. Figure 1 displays the rarefaction curves for the prokaryotic

(A) and eukaryotic (B) rRNA gene sequences, showing an increase in alpha diversity with deeper sequencing for both datasets. In both cases, the curves reach a plateau at higher depths, indicating that the sequencing coverage was sufficient. The prokaryotic dataset exhibited greater diversity compared to the eukaryotic dataset, suggesting a higher species richness or evenness in the bacterial community over time. The sequences were classified at the genus level, the lowest taxonomic rank, using QIIME and the SILVA database, with the results presented in Figure 2.

The observed microbial succession underscores the dynamic nature of spontaneous fermentation, where the initial microbial diversity gradually transitions to communities dominated by key functional microorganisms. In Figure 2A, the initial bacterial community composition was characterized by a high genera diversity, with predominant populations including C*ronobacter, Luteibacter, Erwinia, Agrobacterium, Pseudomonas*, and *Methylobacterium*. The early presence of diverse bacteria likely contributed to the breakdown of complex substrates, setting the stage for the establishment of AAB, LAB, and yeast. These microbial groups are associated with environmental sources, substrates, and human intervention in traditional vinegar production. For example, *Cronobacter* is found in various food matrices, *Luteibacter, Erwinia*, and *Agrobacterium* are plant-associated, and *Pseudomonas, Methylobacterium*, and S*phingomonas* are abundant in apple ecosystems (Ashfaq et al., 2022; Cechin et al., 2023; Iguchi et al., 2023; Wassermann et al., 2019; Zeng et al., 2021).

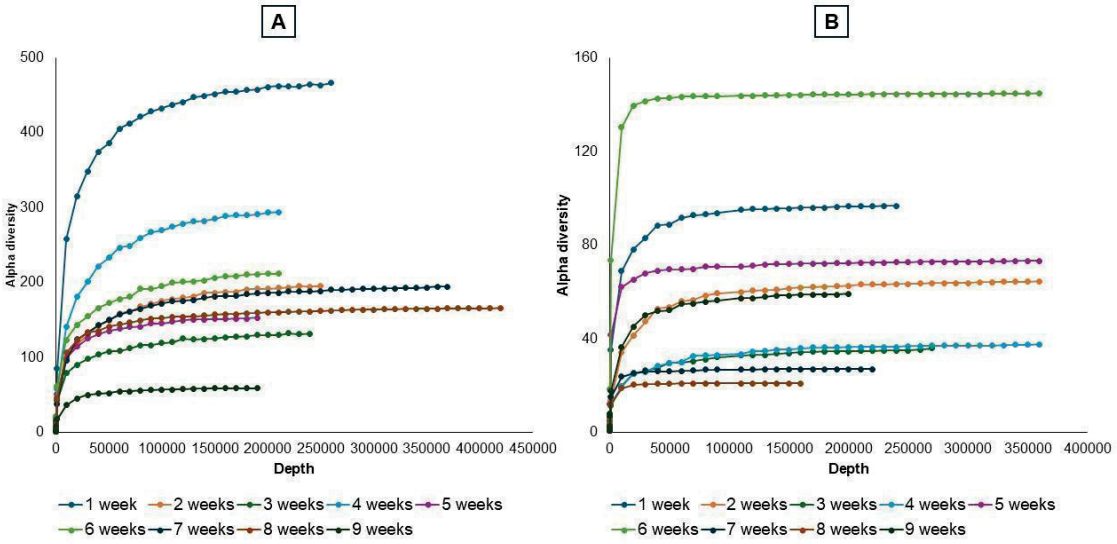


Figure 1. Rarefaction curves for 16S rRNA (A) and 18S rRNA (B) gene sequences obtained from NGS analysis of microbial samples collected over a three-month vinegar fermentation period.

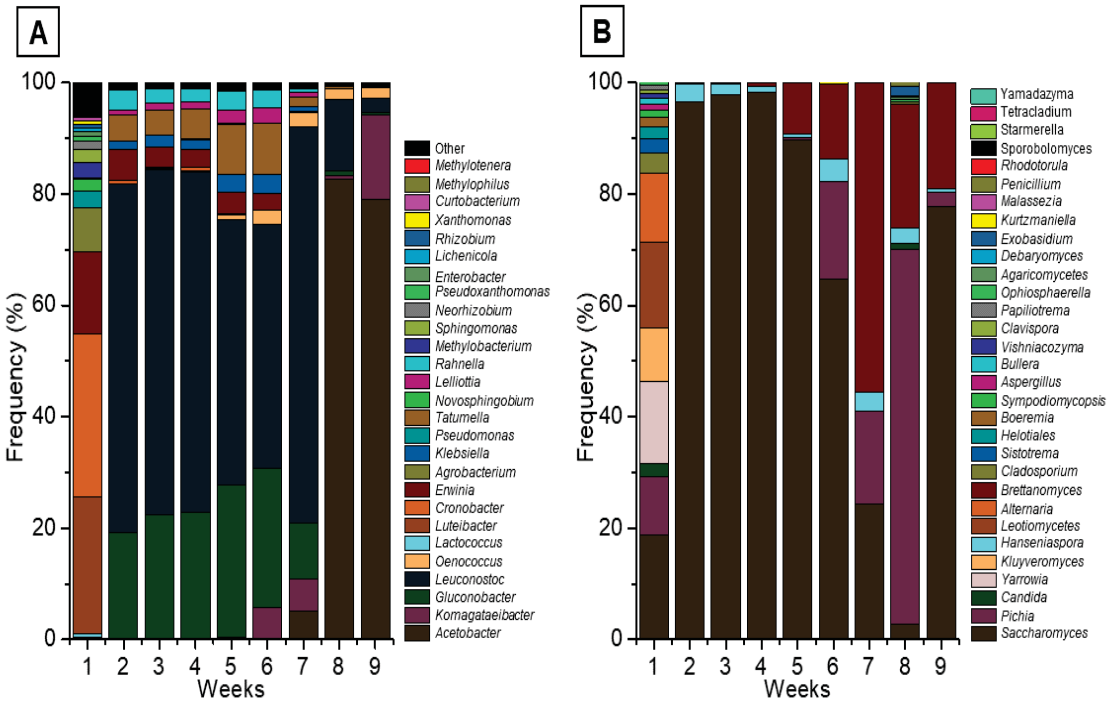


Figure 2. Microbial dynamics during vinegar fermentation analyzed using Illumina amplicon sequencing for bacteria (A) and fungi (B). The bar plots represent the relative abundance of microbial taxa across nine fermentation weeks, illustrating the succession and dominance of key groups involved in the process. The complete list of microbial groups is reported in the ANNEXE (Table A3).

As fermentation progressed, the bacterial community underwent significant changes. From weeks two to seven, *Leuconostoc* dominated the bacterial community, indicating its crucial role in the mid-stages of fermentation. This genus plays a key part in shaping the fermentation environment by producing lactic acid, which lowers the pH and creates conditions favorable for subsequent microbial activities (Roșca et al., 2024). Although *Leuconostoc* is a common genus in vinegar fermentation (Gan et al., 2017), it is typically less prevalent than other LAB such as *Lactobacillus casei* (recently reclassified as *Lacticaseibacillus casei*) (Sengun et al., 2022). However, this is the first documented case where *Leuconostoc* has been found in higher concentrations than *Lactobacillus* during the traditional fermentation of apple vinegar. This discovery suggests a potentially significant shift in microbial dynamics, highlighting the importance of *Leuconostoc* in the development of flavor and quality characteristics that may have been previously underestimated in apple vinegar fermentation. Furthermore, *Leuconostoc* species secrete bacteriocins and other antimicrobial compounds—such as acetic acid, phenyllactic acid, and hydroxyphenyllactic acid—which inhibit spoilage microorganisms and pathogens, contributing to microbial stability (Darbandi et al., 2022). This finding highlights the potential role of *Leuconostoc* in shaping microbial dynamics and contributing to unique flavor profiles in apple vinegar, suggesting that it may play a more influential role than previously recognized.

Gluconobacter was prevalent from weeks two to seven, contributing to the oxidative conversion of sugars into organic acids, lowering the pH, and shaping the fermentation profile (da Silva et al., 2022). Commonly found on harvested apples, pomace, and juice (Hommel, 2014), it plays a key role in vinegar production by oxidizing sugars to acids without complete breakdown, enhancing the acidity and flavor (Leitão et al., 2023). This genus also produces gluconic acid, keto gluconates, and bioactive compounds like riboflavin (B2), improving both product quality and microbial stability (Hata et al., 2023; Noman et al., 2020). *Gluconobacter* species, including *G. japonicus,* are found in the early fermentation stages of various vinegars such as Persian date vinegar (Nosratabadi et al., 2024). Its versatile contributions underscore its importance in enhancing the vinegar quality and health benefits.

In the final stages (weeks eight to nine), *Acetobacter* became dominant, representing over 80% of the reads. Known for its essential role in vinegar production, *Acetobacter* oxidizes ethanol into acetic acid, the primary component of vinegar. Its efficient metabolism enables it to thrive in acidic environments with high ethanol levels, ensuring the complete conversion of alcohol into acetic acid and finalizing the fermentation process. Additionally, *Komagataeibacter* is 15.23% of total reads in the final week. This genus is recognized for its efficient acetic acid production and robust biofilm formation, which contribute to the stability and quality of the vinegar product. *Komagataeibacter* species are known for their ability to produce cellulose, creating a thick biofilm that protects the bacteria and helps maintain optimal fermentation conditions. This biofilm formation is crucial for the continuous and consistent production of acetic acid, as it helps in maintaining the bacterial population in a stable state, ensuring a high yield and quality of vinegar. Furthermore, *Komagataeibacter* can tolerate high acetic acid concentrations, making it highly effective in the latter stages of fermentation when acetic acid levels are at their peak (Gomes et al., 2018; Lin et al., 2020). European studies on vinegar fermentation reveal a trend of *Komagataeibacter* dominating in red wine vinegar, while in apple vinegar, both *Komagataeibacter* and *Acetobacter* are typically balanced throughout the process (Seo et al., 2022; Trček et al., 2016). However, in the present study, *Acetobacter* was unexpectedly far more prevalent than *Komagataeibacter*. This shift in microbial dominance suggests that environmental factors, fermentation conditions, or substrate composition may favor *Acetobacter* over *Komagataeibacter*, potentially altering the fermentation dynamics and influencing the final product's acidity and flavor profile.

Figure 2B presents the fungal community succession over a nine-week fermentation period. Initially, a diverse array of fungi genera was observed, including *Saccharomyces* (18.77%), *Leotiomycetes* (15.52%), *Yarrowia* (14.68%), *Alternaria* (12.22%), *Pichia* (10.54%), *Kluyveromyces* (9.52%), *Cladosporium* (3.76%), *Sistotrema* (2.41%), *Helotiales* (2.27%), *Boeremia* (1.76%), *Candida* (2.41%), *Sympodiomycopsis* (1.16%), and *Aspergillus* (1.11%). As fermentation progressed, *Saccharomyces* became increasingly dominant, maintaining a frequency of over 70% from the second week of fermentation.

Saccharomyces plays a crucial role in the alcoholic fermentation phase, converting sugars into ethanol, which serves as a precursor for acetic acid production by AAB (Luzón-Quintana et al., 2021). This yeast exhibits superior competitiveness in the fermentative environment due to its tolerance to high ethanol concentrations, efficient sugar metabolism, and ability to thrive under low oxygen conditions (Parapouli et al., 2020). Additionally, *Saccharomyces* contributes to the flavor profile by generating esters and alcohols during fermentation, which enhances the sensory qualities of the final product (Nosratabadi et al., 2024). Thus, the careful selection of yeast strains for mixed cultures is critical to vinegar fermentation, as it directly influences the balance between alcoholic and acetic fermentations, ultimately shaping the beverage's quality. Yeast strains can also modulate the interplay between acetic acid fermentation and lactic acid production, further affecting the complexity and stability of the final product (Kim et al., 2021).

Brettanomyces and *Pichia* emerged in the final stages of the fermentation process (Figure 2). *Brettanomyces* are known for producing a range of volatile compounds, including phenolic compounds such as 4-ethylphenol and 4-ethylguaiacol, which can impart complex flavors like smokey, spicy, or barnyard note (Tubia et al., 2018). While these compounds add depth and character in controlled amounts, an excess of *Brettanomyces* activity can result in off-flavors, making its presence a double-edged sword, valuable in moderation but potentially detrimental if overexpressed. *Pichia*, on the other hand, have been identified in organic apple cider vinegars, showing resistance to acetic acid concentrations up to 12 g/L (Luzón-Quintana et al., 2021; Parapouli et al., 2020; Tubia et al., 2018; N. Wang et al., 2023). Their acid tolerance and persistence during fermentation promote high ester production, contributing to the creation of a flavorful vinegar.

Substrates and Metabolites

The observed dynamics of the HPLC analysis of the compounds revealed distinct metabolic patterns within the vinegar fermentation process (Figure 3). Glucose and fructose from supplemented sugar, along with apple-derived fructose, gradually decreased across sampling points, reflecting their utilization by yeasts and LAB for energy and biosynthesis.

By the end of fermentation, their concentrations were 44.28 and 5.77 g/L, respectively, indicating that fructose was the preferred substrate for consumption. A declining trend suggests the conversion of glucose and fructose in two different moments. Glucose was sharply consumed until week three, after which its consumption stabilized until the end of fermentation. This is likely due to most microorganisms being glucophilic and preferentially utilizing glucose to convert into final metabolites. However, fructose was drastically consumed from week five onwards, resulting in low residual levels by the end of the vinegar fermentation. This is consistent with the fact that the genus *Acetobacter,* which utilizes fructose for cellulose production, begins to appear in the Illumina analysis in weeks six to seven, reaching nearly 80% dominance by the end of fermentation (Avirasdya et al., 2022).

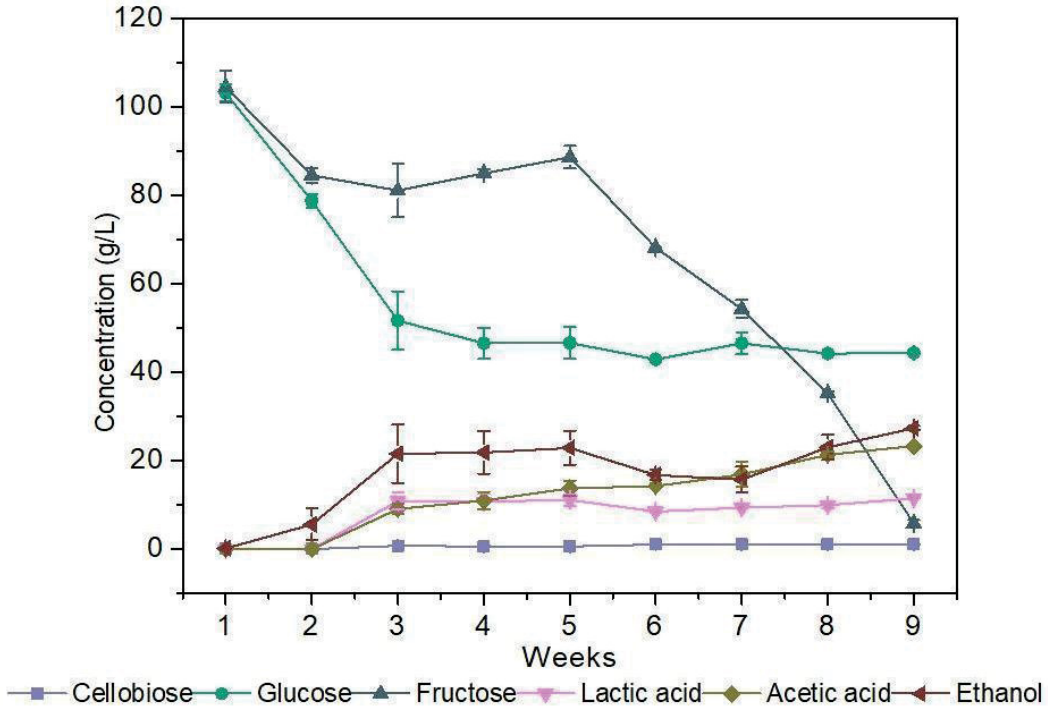


Figure 3. Sugar consumption and organic acids production (g L^{-1}) during a three-month vinegar fermentation.

Lactic acid production increased with the rise of the LAB populations, peaking at 11.48 g/L by the end of 10 weeks, followed by a plateau. This plateau may indicate that the LAB populations stabilized, continuing to metabolize available glucose and fructose while other microbial groups began contributing to fermentation dynamics. The early and continuous presence of LAB reflects their pivotal role in establishing favorable conditions, such as pH reduction, that shape the microbial community throughout the process.

Ethanol levels steadily increased from the start of fermentation, peaking at week eight, reflecting continuous microbial activity and effective sugar conversion despite fluctuations in substrate availability. The high ethanol concentration at this stage is essential, as it serves as a precursor for acetic acid production by the AAB during the subsequent acetous fermentation. The presence of ethanol throughout the process also highlights the persistence of fermentative yeast, which play a critical role in sustaining the microbial community dynamics.

By week nine, ethanol levels declined as the AAB oxidized it into acetic acid, which accumulated progressively throughout the acetous phase, peaking at 23.30 g/L. This gradual increase in acetic acid confirms the efficient conversion of ethanol, ensuring the completion of vinegar fermentation. Notably, the acetic acid concentration remained moderate compared to other vinegar fermentations, such as persimmon vinegar, where acetic acid levels surpass 40 g/L (Wang et al.,2022), a concentration lethal to sensitive species like *Acetobacter* and *Komagataeibacter*.

The moderate acidity in the present fermentation supported the persistence of both *Acetobacter* and *Komagataeibacter,* with *Acetobacter* showing a particular advantage under these conditions. This lower acetic acid concentration fostered microbial diversity, ensuring that sensitive species could thrive and contribute to the fermentation process, leading to a more complex microbial ecosystem.

The dynamic interplay between glucose and fructose utilization, ethanol production, and acetic acid accumulation reflects the intricate balance of metabolic pathways. The effective management of substrate availability and fermentation conditions is essential to maintain microbial diversity, optimize product quality, and ensure stability throughout the process. These interactions highlight the importance of precision in fermentation to achieve a desirable balance between microbial activity and product attributes.

Volatile Compound Formation

The HS-SPME/GC analysis identified 56 volatile compounds, including 17 esters, 14 alcohols, 13 carboxylic acids, 6 aldehydes, 3 ethers, 2 alkanes, and 1 ketone (Figure 4). The results are expressed semi-quantitatively based on the peak area, which reflects the relative abundance of each compound. The sample underwent two distinct fermentation phases. In the initial alcoholic fermentation, glucose was converted into ethanol by yeasts present in the microbiota, as expected (Nie et al., 2017). This was followed by acetic fermentation, during which acetic acid bacteria (AAB) transformed ethanol into acetic acid. This sequential fermentation not only produced a variety of volatile compounds but also resulted in the consumption or bioconversion of other substances, contributing to the complexity and depth of the final product.

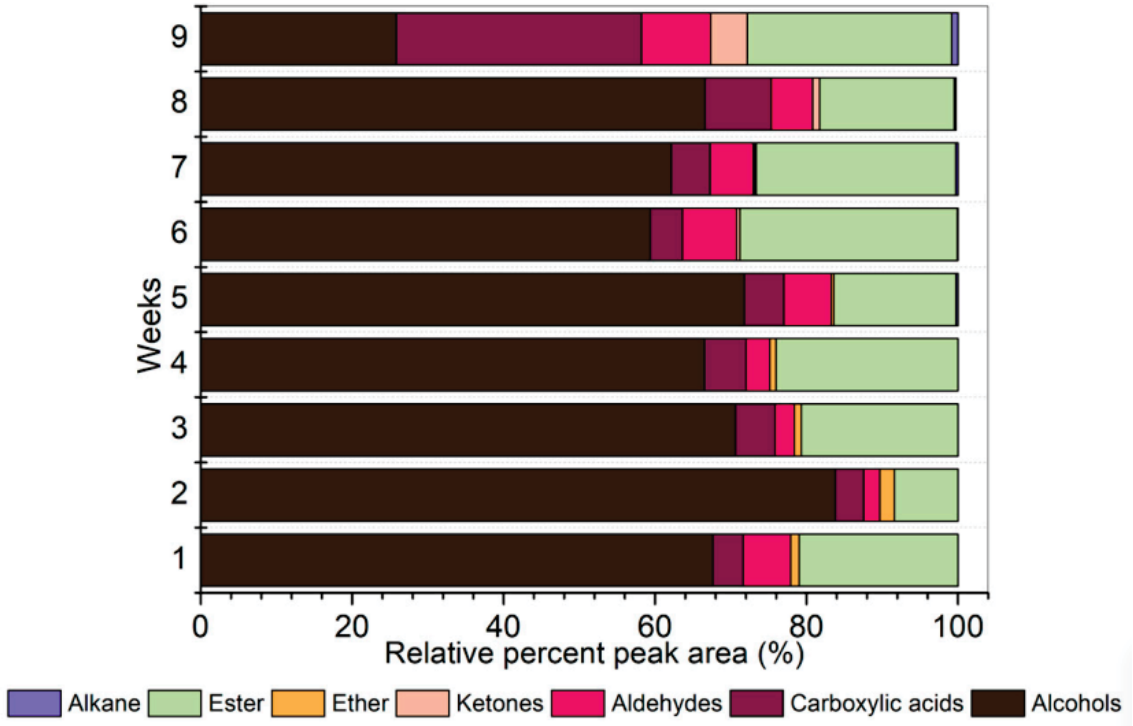


Figure 4. Volatile compounds (area×10^5) identified in apple vinegar fermentation by GC-MS analysis.

At the beginning of fermentation (Table 1), aldehydes, alcohol, and esters were detected. Aldehydes and alcohol were particularly prevalent in the early stages due to alcoholic fermentation. Alcohols are synthesized through the primary and secondary metabolism of yeasts, such as the Shikimate Pathway, which is responsible for the biosynthesis and conversion of aromatic amino acids into various compounds (Averesch and Krömer, 2018). Phenethyl alcohol, a higher alcohol with a rose-like aroma, is produced by the Shikimate Pathway and exhibited the highest amount (703) among the alcohols at the onset of fermentation. This can be correlated with the dominance of *S. cerevisiae*, which is known for producing this compound. The majority of alcohols act as precursors for other compounds and are subsequently consumed over the course of the fermentation process.

Table 1. GC-MS profile aroma of apple natural vinegar (area $\times 10^5$).

Compound	Odor	Taste	Beginning (Week 1)	Middle (Week 5)	End (Week 9)
Carboxylic acids					
Octanoic acid	Faint/Fruity acid	Slightly sour	26,177	24,157	41,033
4-Terpineol	Pine	Herbal pepper	18,017	13,141	0
Nonanoic acid	Fatty	Coconut	0	3586	13,084
Butyric acid	Rancid	Butterfat	0	0	66,785
Isovaleric acid	Rancid-cheesy	Acid	0	0	57,223
Caproic acid	Characteristic goat-like	ND	0	0	14,583
Aldehydes					
2,4-dimethyl Benzaldehyde	Bitter almond	ND	115,062	129,278	53,130
Decanal	Floral-fatty/citrus	Sharp orange	5552	6717	4844
Nonanal	Orange-rose	ND	4406	7.5	5698

Benzaldehyde	Almond oil	Burning aromatic/Bitter almond	0	0	27,199
Ketones					
Acetoin	Buttery	Fatty creamy	0	10,032	48,329
Alcohols					
Phenylethyl alcohol	Rose-like	Initially bitter then sweet/Reminiscent of peach	703,008	652,024	76,180
Isoamyl alcohol	Disagreeable	Pungent/Repulsive	361,700	338,652	3310
1-Butanol	Harsh fusel with banana	Banana/Fusel	51,492	39,933	0
1-Hexanol	Sweet alcohol	Fatty/Fruity	34,249	32,602	0
Isoamyl acetate	Pear-like	Bittersweet reminiscent of pear/Slight apple	24,254	24,714	0
Benzyl alcohol	Faint aromatic	Sharp burning	23,079	19,076	0
2,3-Butanediol	Odorless	Sweet	136,944	0	0
2-Ethyl-1-hexanol	Mild/Oily/Sweet/Floral/Reminiscent of rose	Sweet/Fatty-floral/Fruital note	4458	5116	40,784
Ether					
Estragole	Reminiscent of anise	Sweet	13,933	0	0
Benzene	Aromatic	ND	8537	0	0
Ester					
Phenethyl	Fruity	Flower/Honey/Rose	165,065	171,126	52,570

acetate					
Ethyl palmitate	Waxy	ND	115,960	116,372	37,692
Ethyl decanoate	Oily brandy-like	Brandy/Grape/Pear	70,352	109,201	0
Ethyl octanoate	Wine/Brandy/Fruity/Floral	Apricot/Brandy/Fat/Floral/Pineapple	32,700	74,141	0
Ethyl hexanoate	Wine-like	Apple Peel/Brandy/Fruit Gum/Overripe Fruit/Pineapple	7287	12.9	0
Ethyl dodecanoate	Fruity/Floral	Floral/Fruit/Leaf	0	50,400	0
Ethyl tetradecanoate	Waxy/Reminiscent of orris	Wax	0	17,668	8526
Ethyl butyrate	Banana/Pineapple	Sweet/Pineapple	0	0	16,697

ND: not detected

Aldehydes are formed from the oxidation of the alcohols and fatty acids present in apples. 2,4-dimethyl benzaldehyde, an aldehyde with a bitter almond aroma, exhibited its highest amount (115) at the beginning of fermentation and its lowest amount (53) at the end. This compound is not typically associated with microorganisms or fruits. Conversely, benzaldehyde was observed only at the end of fermentation. This almondlike aldehyde is produced by certain yeasts, such as *Saccharomyces cerevisiae* and *Candida* spp., as the byproduct of alcoholic fermentation, especially under stress conditions. Long Term fermentation can elevate stress within microorganisms due to several factors, such as metabolite accumulation and nutrient limitation, which can explain the increase in benzaldehyde levels only during the final stages of fermentation (Tang et al., 2022).

Esters that enhance the aromatic profile of vinegar can be formed during fermentation or occur naturally in apples. These compounds are completely or partially consumed during the fermentation process, serving as precursors for other volatile compounds. Phenethyl acetate (165), ethyl palmitate (116), and ethyl decanoate (70) were the main esters at the beginning of fermentation. Phenethyl acetate, which has a pleasant fruity and floral aroma, is produced by the conversion of phenyl pyruvic acid in the Shikimate Pathway, primarily through the activity of *S. cerevisiae* (Shende,Vikram V. Bauman, Katherine D. and Moore, 2024). Phenethyl acetate and isoamyl acetate have been detected during apple vinegar fermentation across multiple regions, including China, Japan, and Spain (del Fresno et al., 2022; Ji, 2022; Song et al., 2019). These esters contribute significantly to the aromatic profile of the final product, imparting fruity and floral notes essential for high-quality vinegar.

Ethyl palmitate and ethyl decanoate are esters formed through the reaction between palmitic acid and decanoic acid (present in apple pulp) and ethanol and are produced by yeasts during fermentation. These reactions are facilitated by microbial lipases, which catalyze the esterification process, even though the esters are not directly synthesized by the microorganisms. Towards the end of fermentation, an increase in ethyl butyrate—an ester with distinct banana and pineapple aromas—was observed. This compound is linked to both yeasts and acetic acid bacteria from the Acetobacter genus, which exhibits significant activity in the later fermentation stages.

The delayed accumulation of this ester could be due to the late production of butyric acid, a precursor with a rancid-like aroma that plays a critical role in its formation (Shu et al., 2011). This interplay between microbial metabolism and ester formation highlights the complexity of fermentation, where timing and substrate availability greatly influence the development of the aromatic compounds in the final product.

At the end of fermentation, a decrease in the relative peak areas of most alcohols, ethers, and esters was observed, whereas carboxylic acids, ketones, and some aldehydes exhibited increased peak areas, indicating changes in the volatile profile during fermentation. The carboxylic acids are primarily produced by the oxidation of ethanol by microorganisms (Tomás-Pejó et al., 2023). The main organic acids produced were acetic, butyric, and isovaleric acids. Acetic acid, with a concentration of 12 g/L, is produced by the *Acetobacter* genus through the oxidation of ethanol into acetaldehyde, followed by the oxidation of acetaldehyde into acetic acid. The concentration of acetic acid increases by the end of the fermentation period as *Acetobacter* dominates and suppress other bacteria.

On the other hand, during acetous fermentation, the AAB *Acetobacter* was the genus exhibiting the highest number of strong positive correlations with the compounds in question (Figure 5). The robust positive correlations observed between this genus and the diverse array of compounds suggest a significant metabolic involvement in either the generation or consumption of these substances. Isopentyl alcohol/acetate, recognized for their fruity aromas that are often synthesized during fermentation processes, potentially undergo synthesis or breakdown facilitated by *Acetobacter*, potentially influencing the system's flavor profile (pleasant fruity aroma). Additionally, *Acetobacter*'s correlation with fatty acids, the crucial constituents of cellular membranes and energy sources, hints at its role in lipid metabolism, potentially impacting cell membrane synthesis and energy metabolism within the microbial community. It presented a high correlation to nonanoic acid, providing a cheese and butter flavor. Furthermore, the correlation with 3-cyclohexen-1-ol, a cyclic alcohol with fragrance applications, suggests *Acetobacter*'s potential involvement in its metabolism, potentially influencing the system's aroma profile. Lastly, the strong positive correlation between *Acetobacter* and compounds such as butanoic acid, ethanol, phenol, caproic acid, and benzaldehyde, known for various industrial applications and as intermediates in microbial metabolism, underscores *Acetobacter*'s potential involvement in their production, utilization, or transformation within the microbial community.

The AAB *Komagateibacter* has similar metabolite correlations to *Acetobacter*; however, it differs for its stronger correlation to benzaldehyde 2,4-dimethyl, which has a pleasant almond-like aroma.


1
0.5
0
-0.5
-1
Exobasidium
Pichia
Brettanomyces
Methylophilus
Acetobacter
Komagataeibacter
Oenococcus
Hanseniaspora
Aspergillus
Symptodiomycopsis
Candida
Boeremia
Helotiales
Sistotrema
Cladosporium
Kluyveromyces
Alternaria
Yarrowia
Leotiomycetes
Novosphingobium
Sphingomonas
Methylobacterium
Pseudomonas
Agrobacterium
Cronobacter
Luteibacter
Erwinia
Leifsonia
Rahnella
Klebsiella
Gluconobacter
Taiumella
Leuconostoc
Saccharomyces
Succinic acid, diethyl ester
Phenylethyl Alcohol; Benzeneethanol
Acetic acid
Cinnamic acid, methyl ester
Acetic acid, 2-phenylethyl ester
Isopentyl alcohol
Tetradecanoic acid (Myristic acid)
Ethyl 9-hexadecenoate
Decanoic acid (capric acid)
Nonanoic acid (pelargonic acid)
Dodecanoic acid (lauric acid)
3-Cyclohexen-1-ol, 4-methyl-1-(1-methylethyl); p-Menth-1-en-4-ol
Isopentyl alcohol, acetate
Butanoic acid
Ethanol
Phenol, 4-ethyl-2-methoxy-
Caproic acid
Benzaldehyde
Glucose
Acetoin
Caprylic acid
Butanoic acid, 2-methyl- ; Butyric acid, 2-methyl-
Acetic acid, phenylmethyl ester
1-Butanol; sec-Butylcarbinol
Phthalic acid
1-Hexanol
Celobiose
Dodecanoic acid, ethyl ester
Lactic acid
Estragole; Tarragon
2,4-Di-tert-butylphenol
Decanal
Fructose
Cinnamic acid, methyl ester
2,3-Butanediol ; Butane-2,3-diol
2,3-Butanediol
Decanoic acid, ethyl ester; Capric acid, ethyl ester
Thymol
Duraldehyde
Nonanal
Benzaldehyde, 2,4-dimethyl
Octanoic acid, ethyl ester ; Ethyl caprylate
Linalool; 1,6-Octadien-3-ol
Isovaleric acid
Butanoic acid
2-Methyltetracosane
Malonic acid, bis(2-trimethylsilylethyl ester
Benzeneacetic acid, ethyl ester
Benzyl alcohol; Benzenemethanol
2-Ethyl-1-hexanol
Phenol, 4-ethyl
1,3-Dioxolane, 2,4,5-trimethyl
Hexadecanoic acid (palmitic acid)
Butanoic acid, ethyl ester
Ethyl 9-decenoate
Tetradecanoic acid, ethyl ester; Myristic acid, ethyl ester
Propanoic acid, 2-methyl-, 3-hydroxy-2,2,4-trimethylpentyl ester
Hexanoic acid ethyl ester ou Ethyl caproate
Methyleugenol ; Benzene
Heneicosane
Hexadecanoic acid, ethyl ester; Palmitic acid, ethyl ester

Figure 5. Correlation analysis between microbiota, organic acids, and volatile compounds (A) and concentration of volatile aroma compounds (area $\times 10^5$) (B) during vinegar fermentation.

Correlation Analysis

Radar plots illustrating the correlation analysis between microbiota, organic acids, and volatile compounds are presented in Figure 6. During the initial stage of fermentation,

bacteria such as *Leuconoctoc* and *Gluconobacter,* alongside the yeast *Saccharomyces*, showed a strong correlation. It can also be observed that *Saccharomyces* exhibited a negative correlation with lactic acid, which explains its decrease in prevalence throughout the fermentation process. This trend was similarly observed in Chinese traditional Shanxi aged vinegar (Wang et al., 2022). *Leuconostoc* showed moderate correlations (0–0.5) with various compounds, suggesting potential interactions in the vinegar production. Lactic acid, octanoic acid, isovaleric acid, and butanoic acid contribute to flavor and aroma. Benzyl alcohol and 2-ethyl-1 hexanol are known to contribute to the aroma of beverages like wine. Ethyl 9-decenoate and tetradecanoic acid ethyl ester provide fruity aromas, while propanoic acid acts as a preservative compound. Hexanoic acid ethyl ester enhances the fruity aroma of beverages. In addition, it presented a positive correlation to acetoin, similar to other works (Wätjen et al., 2023).

Gluconobacter, unlike *Leuconostoc*, exhibited moderate correlations (0–0.5) with other kinds of compounds, including dodecanoic acid, thymol, 2-methyltetracosane, malonic acid, benzeneacetic acid, butanoic acid ethyl ester, heneicosane, and hexadecanoic acid ethyl ester. Dodecanoic acid may influence the cellular membrane structure and lipid metabolism (Mett and Müller, 2021). Benzeneacetic acid contributes to complex and unique aromas in fermented foods (Jeong et al., 2016). Butanoic acid ethyl ester provides fruity aromas to fermented beverages. Finally, hexadecanoic acid ethyl ester influences aroma and may be involved in the formation of pleasant ester aromas in fermented foods. Additionally, *Gluconobacter* presents a similar correlation as *Leuconostoc* to hexanoic acid, octanoic acid linalool, isovaleric acid, butanoic acid, ethyl 9-decenoate, tetradecanoic acid ethyl ester, propanoic acid, hexanoic acid, and methyleugenol benzene.

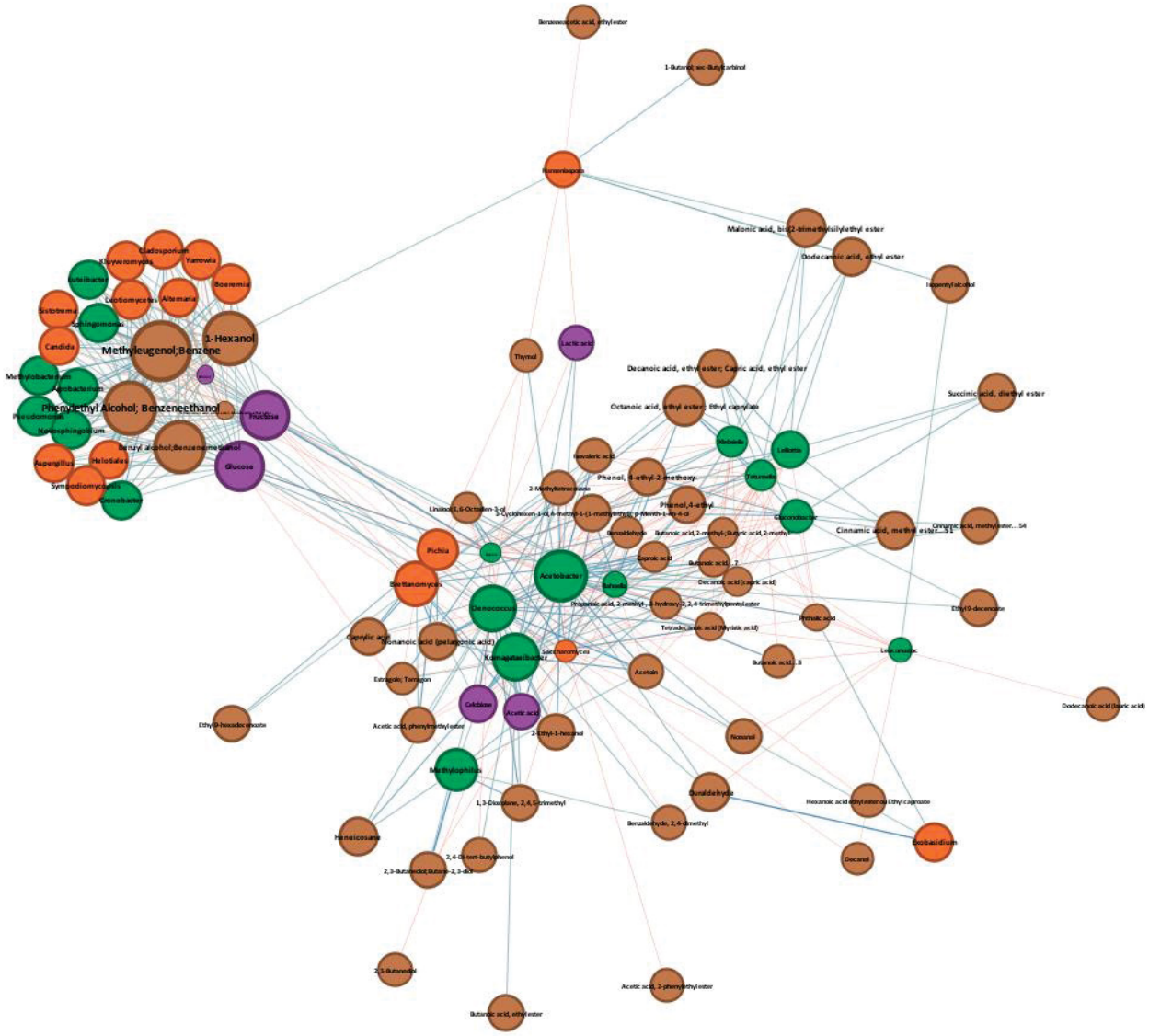


Figure 6. Radar plots of Spearman correlation coefficients showing correlation analysis between microbiota (bacteria in green and fungi in orange) and organic acids, sugar and volatile compounds (brown).

In addition, during acetous fermentation, *Oenococcus* showed a strong correlation to succinic acid diethyl ester, isopentyl alcohol acetate, and benzaldehyde 2,4-dimethyl, representing the LAB to contribute the most flavor to the final vinegar product aroma. *Oenococcus*, a LAB frequently associated with food fermentation, particularly wine production, exhibit a notable capacity for producing compounds such as succinic acid, diethyl ester, isopentyl alcohol acetate, and benzaldehyde 2,4-dimethyl (Diez-Ozaeta et al., 2021). These compounds play multifaceted roles in food fermentation processes. benzaldehyde 2,4-dimethyl. These compounds play multifaceted roles in food fermentation processes. Firstly, isopentyl alcohol acetate (isoamyl acetate), renowned for its fruity aroma akin to banana, and benzaldehyde 2,4-dimethyl, characterized by its

distinctive almond-like scent, contribute significantly to the aromatic profile of the fermented food, enriching it with fruity nuances and sensory intricacies. Secondly, succinic acid and diethyl ester impart specific flavors to the fermented food, enhancing its overall taste and palatability. Additionally, the existence of these aromatic compounds introduces strata of sensory intricacy into the fermented item, thereby augmenting its allure and fascination to consumers. In summary, *Saccharomyces* contributes to alcohol formation, enhancing the fruity aroma. *Leuconostoc* are linked to ester production, enhancing flavor complexity, while *Acetobacter* are associated with acetic acid and VOCs, influencing overall aroma profile.

The correlation analysis revealed that the predominant microbial groups—*Saccharomyces, Gluconobacter, Leuconostoc, Acetobacter, Komagataeibacter,* and *Pichia*—exhibited antagonistic relationships with several undesirable microorganisms, including *Erwinia, Aspergillus, Candida, Pseudomonas*, and *Cronobacter.* These antagonistic interactions suggest that the dominant beneficial microbes help suppress the growth of spoilage organisms, contributing to the microbial stability of vinegar. Furthermore, the undesirable groups showed a negative correlation with acetic acid and lactic acid, reinforcing the idea that higher levels of these organic acids—produced by the key fermentation microorganisms—enhance product safety by creating an unfavorable environment for contaminants. A similar trend was observed in Shanxi aged vinegar (Nie et al., 2017), where a beneficial microbial consortium dominated the fermentation environment, restricting the presence of potential spoilage organisms. The microbial dynamics observed in Shanxi vinegar, which involves a complex multi-stage fermentation, highlight the importance of organic acid production in maintaining the balance between desirable and undesirable microbes. This also underscores the critical role of fermentation management in achieving both microbial safety and enhanced sensory quality in traditional vinegars.

Saccharomyces had a strong correlation with *Leuconostoc, Gluconobacter,* and *Hanseniaspora,* and a weak correlation with *Acetobacter* and *Komagataeibacter.* This is because *Saccharomyces* can utilize carbon sources similarly to *Leuconostoc* and *Gluconobacter* (Turcotte et al., 2010). On the other hand, the weak interaction with *Acetobacter* and *Komagataeibacter* is related to the nature of these microorganisms, which use the ethanol produced by *Saccharomyces* when its growth is reduced (Gomes et al., 2018). *Acetobacter* presented a strong connection with *Komagataeibacter* and *Pichia* due to the high presence of these microorganisms. This interaction is interesting

because while *Acetobacter* produces acetic acid, the metabolism of *Komagataeibacter* and *Pichia* can contribute to the vinegar volatile profile (Hata et al., 2023). It is noteworthy that *Pichia* species can thrive in low pH environments.

Additionally, an intriguing interaction occurs between *Saccharomyces* and *Hanseniaspora* during the mid-stages of fermentation. In wine, for example, the cooperative interaction between both genders in mixed fermentations is characterized by *Hanseniaspora* enhancing the aroma through unique ester production in the initial fermentation phases, followed by *Saccharomyces*'s suppression of *Hanseniaspora* via cell-to-cell contact and competition for essential nutrients, ultimately shaping the final drink profile (Pietrafesa et al., 2020). Thus, the data show that the ideal consortium for complex vinegar fermentation includes the initial co-inoculation of *Leuconostoc, Gluconobacter,* and *Saccharomyces* for the alcoholic phase, followed by the addition of *Acetobacter* and *Komagataeibacter* for the acetic acid phase. Additionally, the addition of *Hanseniaspora* at the initial stage of fermentation and *Pichia* at the final stage can be tested to produce vinegars with more complex and distinctive flavors. These interactions highlight the multifaceted roles of different microbiota in shaping the sensory qualities of traditional fermented vinegar in Brazil.

Conclusions

This study elucidated the microbial dynamics and metabolite profiles during traditional Brazilian apple vinegar fermentation, highlighting the key microbial species and their roles in the fermentation process. Dominant species such as *Saccharomyces, Leuconostoc, Gluconobacter,* and *Acetobacter* were identified as crucial players in the different stages of fermentation. *Saccharomyces* was significant in early alcohol formation, *Leuconostoc* contributed to fruity and floral notes through ester production, and *Acetobacter* were essential in the acetic fermentation stage, enhancing acetic acid production and volatile organic compound formation.

The data suggests that an optimal microbial consortium for vinegar fermentation should include *Saccharomyces* for efficient alcohol production, *Leuconostoc* for ester-mediated flavor complexity, and *Acetobacter* for robust acetic acid production. The presence of *Komagataeibacter* could further improve sensory and functional qualities due to their role in producing bacterial cellulose.

Understanding these microbial interactions and their metabolic pathways is critical for the vinegar industry. This knowledge enables the optimization of fermentation

conditions, improves vinegar quality and consistency, and supports the development of novel vinegar varieties with tailored flavor profiles to meet consumer preferences and market demands. The findings from this study contribute significantly to the advancement of vinegar production practices.

CHAPTER FOUR - VIRUSES IN FERMENTED FOODS: ARE THEY GOOD OR BAD? TWO SIDES OF THE SAME COIN

Manuscript published in *Food Microbiology* journal. DOI: https://doi.org/10.1016/j.fm.2021.103794

Abstract

The emergence of Coronavirus disease 2019 as a global pandemic has increased popular concerns about diseases caused by viruses. Fermented foods containing high loads of viable fungi and bacteria are potential sources for virus contamination. The most common include viruses that infect bacteria (bacteriophage) and yeasts reported in fermented milks, sausages, vegetables, wine, sourdough, and cocoa beans. Recent molecular studies have also associated fermented foods as vehicles for pathogenic human viruses. Human noroviruses, rotavirus, and hepatitis virus have been identified in different fermented foods through multiple routes. No severe acute respiratory syndrome Coronavirus 2 (SARS-CoV-2) virus or close members were found in fermented foods to date. However, the occurrence/persistence of other pathogenic viruses reveals a potential vulnerability of fermented foods to SARS-CoV-2 contamination. On the other side of the coin, some bacteriophages are being suggested for improving the fermentation process and food safety, as well as owing potential probiotic properties in modern fermented foods. This review will address the diversity and characteristics of viruses associated with fermented foods and what has been changed after a short introduction to the most common next-generation sequencing platforms. Also, the risk of SARS-CoV-2 transmission via fermented foods and preventive measures will be discussed.

Keywords: SARS-CoV-2; Rotavirus; Next-generation sequencing; Bacteriophage; Fermented milks.

Introduction

Viruses are ubiquitous in every ecosystem and infect all forms of life, from prokaryotes to eukaryotes (Hyman and Abedon, 2012). Food fermentation is driven by dense microbial consortia consisting mainly of bacteria and fungi (De Melo Pereira et al., 2020). This constitutes a rich reservoir for the development of many microorganism-infecting viruses. Studies have reported the presence of viruses that infect bacteria (bacteriophages) and yeast in plenty of fermented food products, including wine, meat, cheese, yoghurt, sourdough, sauerkraut, kimchi, soybean, and cocoa (Auad et al., 1997; Barrangou et al., 2002; Foschino et al., 2005; Illeghems et al., 2012; Kiliç et al., 1996; Kleppen et al., 2012a; Pringsulaka et al., 2011; Umene et al., 2009). In general, bacteriophages are considered harmful by decreasing the fermentative capacity of lactic acid bacteria (LAB) and yeasts, occasionally resulting in complete fermentation failure. The diversity of phages in fermented foods has been seen to vary according to geography, climate, environment, type of raw material, preparation methods, and microbial composition (Tamang et al., 2020). However, although there is evidence that bacteriophages can cause disease in humans (Tetz and Tetz, 2018), they are not associated with sanitary and public health concerns.

In addition to bacteriophages and yeast-associated viruses, pathogenic viruses have been reported in fermented foods causing injuries and even death (Cho et al., 2016; Colson et al., 2010; Holzmann et al., 2009; Hossain et al., 2016). The zoonotic Nipah virus (NiV), for example, was attributed as the probable cause of the death of eight victims who ingested a traditional fermented liquor in Bangladesh (Hossain et al., 2016). Viruses have high environmental resistance being able to survive against microorganisms' elimination processes (Vasickova et al., 2010), using these products as vehicles to human contagion. Currently, human noroviruses (NoVs) are recognized as the main cause of viral foodborne outbreaks, followed by rotavirus (RV) and hepatitis virus (HV) (Leblanc et al., 2019). Contamination can occur in different ways, mainly through infected raw materials and improper food handling. RVs cause an estimated 111 thousand cases of diarrhea per year, two million hospitalizations, and 400 thousand fatalities in children under five years old, where over 80% of the registered cases come from undeveloped countries (Food and Agriculture Organization and World Health Organization, 2009; Tamang et al., 2020). The emergence of Coronavirus disease 2019 (COVID-19) as a global pandemic has increased popular concerns about diseases caused by viruses (Lai et

al., 2020). Despite the principal form of spreading is human-to-human contact, the COVID-19 similar Middle East Respiratory Syndrome (MERS-CoV) virus remains infectious up to 60 min outside the body (Pyankov et al., 2018). Security authorities raised concern on viral transmission by food, as preparation and delivery can be critical steps on transmission (Rizou et al., 2020).

The presence of viruses in fermented foods has traditionally been studied by culture-dependent methods. These methods are focused on singular bacteriophages that cause fermentative flaws and on pathogenic human viruses (Park et al., 2011). However, with the advancement of molecular techniques and the outgrowth of next-generation sequencing (NGS), a large body of metagenomic sequencing information was allowed, circumventing the need for gene cloning or cultivation (Vinicius De Melo Pereira et al., 2020). Fermented food microbiomes have been accessed, and what is known as "viromes" has emerged. However, the number of virome studies still lags far behind that of bacteria and fungi publications; for each virome in 2019, there were 42 microbiome studies (Ledormand et al., 2020; Tamang et al., 2020).

The lack of a universal molecular marker, as the 16S and 18S rRNA in bacteria and fungi, respectively, can be considered an obstacle for virome studies in food matrices. Nevertheless, shotgun metagenomic, which does not depend on a target ribosomal marker, has successively characterized viral communities from freshwater, soil, ocean, mammalian gut and, to a lesser extent, fermented food products (Dugat-Bony et al., 2020; Hayes et al., 2017; Park et al., 2011). Recently, pyrosequencing and Illumina Miseq platforms have been used to characterize viral communities of different fermented foods (Dugat-Bony et al., 2020; Jung et al., 2018). NGS viromes confirmed the dominance of Caudovir ales bacteriophages as on culture-dependent approaches.

NGS studies have enabled new applications and perspectives for bacteriophages and, thus, accessing the other side of the coin. It has been observed that some bacteriophages can modulate bacterial community succession during the fermentation process, positively affecting food quality and sensorial properties (Agyirifo et al., 2019). Quorum sensing studies have not yet been accomplished to better understand this modulation and relationship. Gastrointestinal tract (GIT) bacterial community is also shaped by bacteriophages contained in fermented foods, impacting host physiology and metabolism. They are able to trigger immune responses through direct contact with mucosal epithelial cells (locally) or with immune system components (systemically)

(Sausset et al., 2020). These features enable bacteriophages to provide probiotic effects, as suggested by Pacini and Ruggiero, (2019), through the ingestion of phage-containing fermented milk and colostrum.

This review will provide a general overview of viruses associated with fermented foods and what has been changed after a short introduction to the most common NGS platforms, as well as a critical discussion on the potential of fermented foods to deliver viruses with public health concerns. Additionally, NGS strategies and methods for describing food viromes will be addressed with the ultimate objective of assisting future evaluation studies.

Bacteriophages and yeast viruses

Bacteria comprise a highly diverse group, representing the second major biomass element on Earth (~15%), behind only plants (~80%). This abundant living mass is a huge reservoir for bacteriophage (or simply called phage) predation. As phages can be found repeatedly in different hosts, they represent approximately 1031 particles, ten times the number of bacteria (1030 cells) (Breitbart and Rohwer, 2005). Bacteriophages are small in size (isometric heads are typically 45–170 nm in diameter) and are composed of a single type of nucleic acid with single or double-stranded (ssDNA, dsDNA, ssRNA, dsRNA) protected by a protein or lipoprotein capsid (Orlova, 2012). Phage genomes vary between families ranging from ~3.5 kb (e.g. *Escherichia coli* phage genome) to ~540 kb (*Prevotella* spp. phages genome) (Sausset et al., 2020). They do not have cellular machinery required for transcription, translation, and energy production, using from their hosts. When inside, phage particles are formed and, when bacterial lysis occurs, they are released (lytic cycle). The phages that use only the lytic cycle to propagate are called virulent.

The infection of LAB by bacteriophages is widely investigated to be considered the primary cause of fermentation failure in the dairy industry (Garneau and Moineau, 2011). However, some phages have specific genes to direct their integration into the bacterial chromosome and remain dormant as a prophage until stresses or specific conditions induce the lytic cycle. A bacterial host carrying a prophage is called lysogenic. A temperate phage can form lysogens and initiate either a lytic cycle or a lysogenic cycle, whereas a virulent phage is obligately lytic (Samson and Moineau, 2013a).

Phages have various contamination routes during the manufacture of fermented foods (Figure 1). Eventually, LAB can naturally prevent phage invasion by evolving phage-resistance systems, or it can be genetically engineered to avoid culture devastation (e.g., origin-derived phage encoded resistance, gene silencing, suicide system, and subunit poisoning) (Murphy et al., 2017). The use of physical-chemical methods on materials and industrial facilities also acts in phage contamination prevention. Examples include thermal treatment, use of biocidal agents (sodium hypochlorite and peracetic acid), UV photocatalysis, and high-pressure treatments (high hydrostatic pressure and high-pressure homogenization) (Murphy et al., 2017). However, the existence of resistant viruses allows contamination to still occur (Figure 1). Phage-related issues are not restricted to foods, but also pharmaceutical, chemical, and pesticide industries (Pujato et al., 2019).

A survey on bacteriophages and yeast virus's diversity in fermented foods is reported in Table 1.

Table 1. Bacteriophages and yeast virus diversity in fermented food.

Phage	Fermented food	Reference
Lactobacillus	Yoghurt	(Kiliç et al., 1996); (Auad et al., 1997); (Illeghems et al., 2012)
	Cocoa bean	(Agyirifo et al., 2019)
	Meat	(Trevors et al., 1983)
	Sourdough	(Foschino et al., 2005)
	Sauerkraut	(Barrangou et al., 2002)
	Kimchi	(Jung et al., 2018)
	Fermented cucumber	(Lu et al., 2003a); (Lu et al., 2012)
Lactococcus	Cheese	(Dugat-Bony et al., 2020; Frantzen and Holo, 2019; Gebreselassie, 2014; Mahony et al., 2017; McIntyre et al., 1991; Murphy et al., 2013)
	Fermented milk and colostrum	(Pacini and Ruggiero, 2019)
	Fermented fish	(Phumkhachorn, 2012)
	Kem buk nud	(Phumkhachorn, 2012)
	Kimchi	(Jung et al., 2018)
	Cocoa bean	(Agyirifo et al., 2019)
Leuconostoc	Cheese	(Kleppen et al., 2012b); (Kot et al., 2014); (Dugat-Bony et al., 2020); (Atamer et al., 2011)
	Fermented milk villi	(Saxelin et al., 1986)
	Sauerkraut	(Barrangou et al., 2002); (Lu et al., 2010) (Mudgal et al., 2006)

	Kimchi	(Jung et al., 2011, 2018)
	Fermented pork meat	(Greer et al., 2007)
	Cocoa bean	(Agyirifo et al., 2019)
	Hard cheese and acid curd cheese	(Ali et al., 2013)
	Whey and brine	(Atamer et al., 2011)
	Butter milk and butter cream	(Ali et al., 2013); (Atamer et al., 2011)
Enterococcus	Cheese	(Ladero et al., 2016); (Del Rio et al., 2019)
	Cocoa bean	(Agyirifo et al., 2019)
Streptococcus	Yoghurt	(Bendadis et al., 1990); (Brussow et al., 1994); (Quiberoni et al., 2003); (Ishlimova et al., 2012); (Ma et al., 2014)
	Cheese	(Whitehead and Hunter, 1947); (Brussow et al., 1994); (Quiberoni et al., 2006); (Zinno et al., 2010)
	Fermented milk villi	(Saxelin et al., 1986)
	Fermented milk and colostrum	(Ruggiero, 2019)
Weissella	Kimchi	(Jung et al., 2018); (Kleppen et al., 2012a)
	Fermented pork sausage	(Pringsulaka et al., 2011)
	Fermented cucumber	(Lu et al., 2012)
Oenococcus	Wine	(Doria et al., 2013)
Bacillus	Natto	(Umene et al., 2009); (Nagai and Yamasaki, 2009); (Umene and Shiraishi, 2013)
	Doenjang, Jangajji, Meju and Gochujang	(Shin et al., 2011)
	Kinema	(Kumar et al., 2019)
	Cocoa bean	(Agyirifo et al., 2019)
Staphylococcus	Kimchi	(Jung et al., 2018)
	Cocoa bean	(Agyirifo et al., 2019)
	Salami	(Bruttin et al., 1992)
Enterobacter	Cocoa bean	(Illeghems et al., 2012); (Agyirifo et al., 2019)
Pseudoalteromonas	Cheese	(Dugat-Bony et al., 2020)
Vibrio	Cheese	(Dugat-Bony et al., 2020)
Halomonas	Cheese	(Dugat-Bony et al., 2020)
Propionibacterium	Cheese	(L. Cheng et al., 2018)
Klebsiella	Cocoa bean	(Illeghems et al., 2012)
Pseudomonas	Cocoa bean	(Agyirifo et al., 2019)
Gluconobacter	Wine	(Philippe et al., 2018)
Yeast virus		
Saccharomyces cerevisiae	Wine	(Rodríguez-Cousiño et al., 2011a)

They are mainly represented by LAB phages (*Lactococcus, Lactobacillus, Leuconostoc, Enterococcus, Streptococcus*, and *Weissella*), being *Lactococcus*, *Lactobacillus,* and *Leuconostoc* most reported in fermented foods (De Melo Pereira et al., 2020). Bacteriophages infecting LAB are all members of the *Caudovirales*, an order known as the non-enveloped dsDNA tailed phages. *Caudovirales* use the tail section to bind to the receptor on the bacterial cell wall, and the genome passes down the tail into the bacteria cell. Once inside, the virus genome is replicated by overlapping bacterial DNA and replicating the complete viral genome. Three families within the *Caudovirales* order, i. e., *Myoviridae, Siphoviridae*, and *Podoviridae*, were reported in fermented foods (Figure 1). *Myoviridae* are characterized by the presence of a long contractile tail, *Siphoviridae* has a long, non-contractile tail, and *Podoviridae* a short, non-contractile tail (Samson and Moineau, 2013b). This classification, which is based on morphology instead of DNA sequences, originated by Bradley in 1969 and has been extended to date.

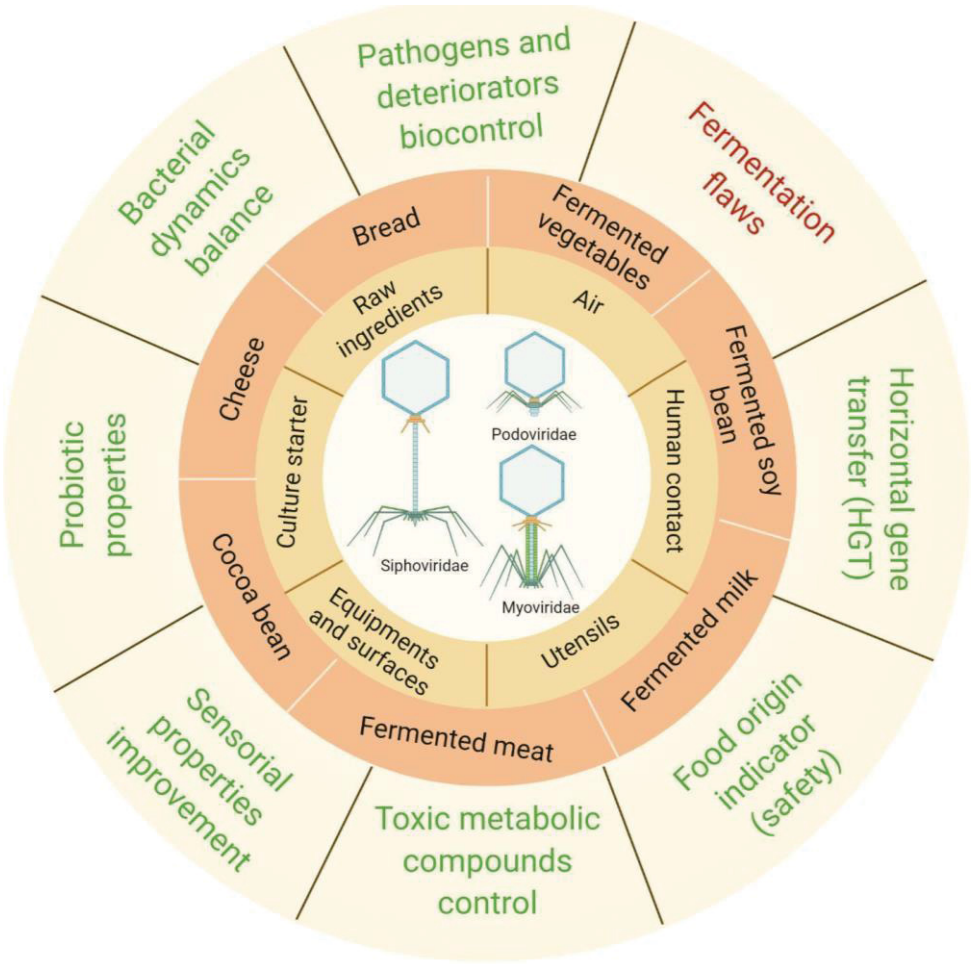


Figure. 1. Main bacteriophages families and contagion routes associated with fermented foods (adapted from Samson and Moineau, 2013). Positive associations are written in green and negative in red on the

outer part of the circle. This figure was created using BioRender (https://biorender.com/). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

The first LAB phage report was published 85 years ago, a streptococci attack-phage in cheese (Whitehead and Hunter, 1947). Posteriorly, the presence of phage was widely reported during lactic fermentation (e.g., sauerkraut, yogurt, natto, and cucumber) (Barrangou et al., 2002; Brussow et al., 1994; Kiliç et al., 1996; Kleppen et al., 2012b; Lu et al., 2003b; McIntyre et al., 1991; Quiberoni et al., 2003; Saxelin et al., 1986; Umene and Shiraishi, 2013). Phage attack was frequently associated with fermentation failure until complete loss of the product batch due to infeasibility of starter cultures (Quiberoni et al., 2003). Starter-destroying phages are not restricted to LAB. Other genera that compose starter cultures are also attacked by phages, such as *Bacillus* spp. in fermented soybean known as natto (Umene et al., 2009), *Staphylococcus* spp. in salami (Bruttin et al., 1992), and even on yeast (*S. cerevisiae*) as called 'yeast viruses' in winemaking (Ramírez et al., 2015; Rodríguez-Cousino et al., 2011). Different from bacteriophages, *S. cerevisiae* viruses belong to the *Totiviridae* family (*Ghabrivirales* order) and infect not only yeasts but also protozoa, filamentous fungi, plants, invertebrates, and vertebrates (Rowley, 2017).

The presence of bacteriophages in microbial cultures is not sufficient for affecting fermentation processes. It depends on the microbial composition in the starter, being the more diverse the composition, the less chance of failure (Spus et al., 2015). This can be explained by the fact that bacteria have different levels of sensitivity to phages and, when an attack occurs, some bacteria are resistant to recover from fermentation. Beyond that, contamination seems to be attributed to the consistency of the food matrix in which liquid matrices are more susceptible to allowing a rapid phage to spread than solid or semi-solid. Bruttin et al.,(1992) attributed the solid-state of meat in salami as the key factor to prevent attack by *Staphylococcus carnosus* bacteriophages, while liquid milk fermentation allows easiest phages propagation (Garneau and Moineau, 2011).

Human foodborne and zoonotic viruses

Food fermenters produce different end-metabolites that have antiviral activity, including bacteriocins, hydrogen peroxide, ethanol, and lactic acid. However, some enteric viruses, including NoV, hepatitis A virus (HAV), hepatitis E virus (HEV), and Orthopoxvirus and Henipavirus genus, can remain viable in foods for periods from two days to four weeks (Hewitt and Greening, 2004), even in an environment with the lack of

specific host cells to replicate. Contamination routes include (i) the raw material contaminated before food preparation, (ii) food preparation and processing, emphasizing the role of food handler, hygienic conditions, and the use of polluted materials, and (iii) food delivery and facilities routes where the food passes after it is finished (Figure. 3).

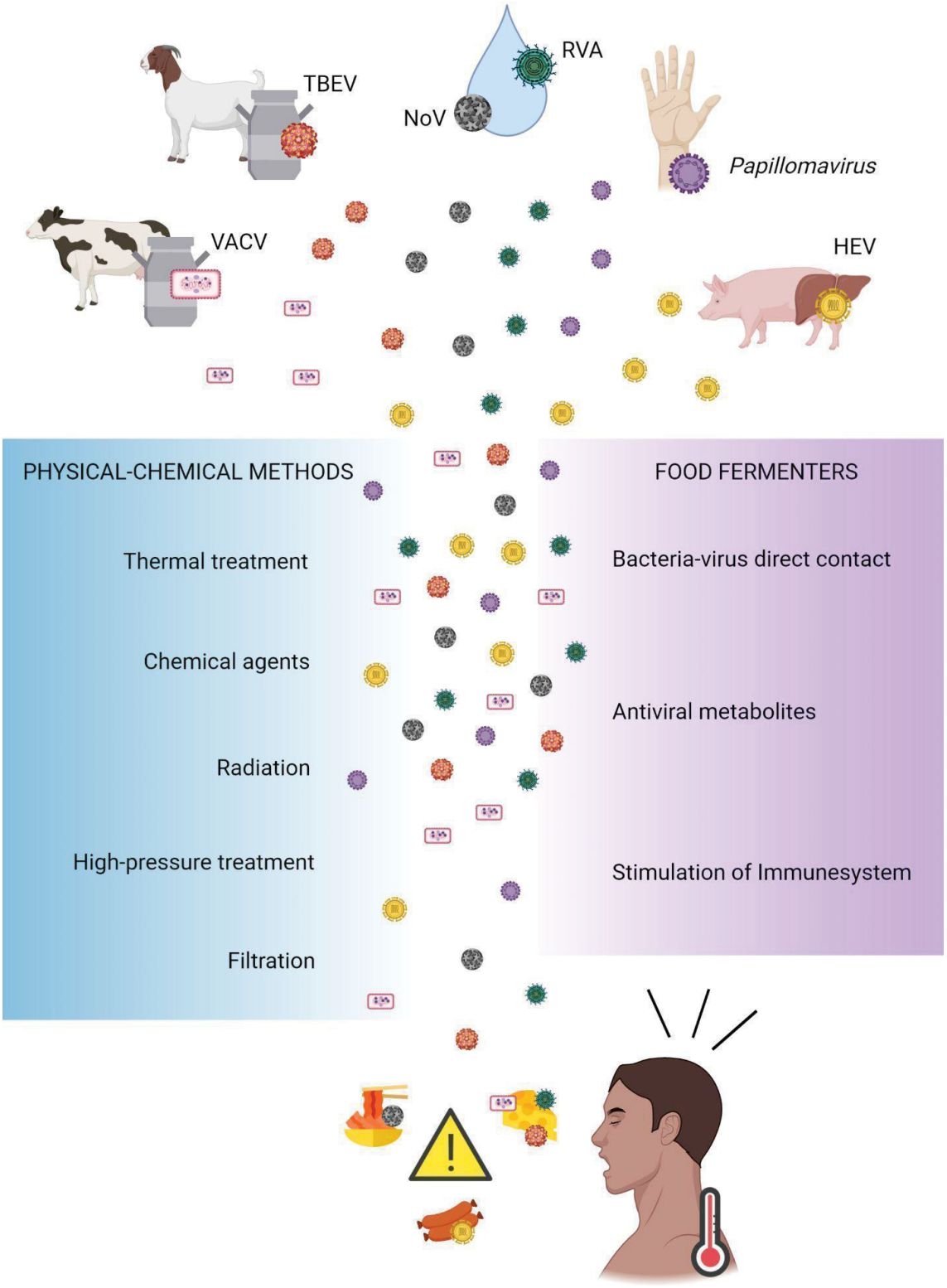


Figure 3. Main sources of pathogenic viruses and prevention measures associated with fermented foods. VACV (vaccinia virus); TBVE (tick-borne encephalitis virus); NoV (Norovirus); HEV (hepatitis E virus); RVA (rotavirus A). This figure was created using BioRender (https://biorender.com/)

Tick-borne encephalitis virus (TBEV) outbreaks were associated with cheese made with infected goat's milk in Europe (Brockmann et al., 2018; Holzmann et al., 2009; Markovinović et al., 2016). Contaminated milk with vaccinia virus (VACV) during cheese production in Brazil has also been reported (Rehfeld et al., 2017). VACV is considered a zoonosis affecting cows and humans. Artisanal cheese produced with non-pasteurized milk increases the chance of infection. Rehfeld et al., (2017) found that VACV remains viable after 60 days of cheese ripening at 25 ◦C. Meat products are also potential targets for viral contamination, giving special attention to HEV due to its zoonotic potential (Colson et al., 2010). The target cells of the virus are hepatocytes; therefore, the greatest risk is the consumption of contaminated animals' liver. It was recently found that, even in pH 2, there were remaining infectious virus particles in fermented meat products (Wolff et al., 2020).

Fermented oyster and kimchi were associated with NoV produced with polluted water in South Korea (Park et al., 2015). NoV has high infectivity, having the ability to withstand a broad range of temperatures and high resistance to acidic conditions. These factors facilitate NoV transmission (Park et al., 2015). Bae et al., (2018) and Gagné et al., (2015) evaluated NoV survival in experimentally contaminated kimchi and sauerkraut, respectively. In extended periods of fermentation by 90 days, virus load has decreased; however, viable copies were still present at the end of the fermentation. Rotavirus A (RVA) is also easily spread by polluted water or sewage. Recently, de Castro Carvalho et al., (2020) reported RVA in homemade Minas Frescal cheese collected from local markets in the city of Mariana, Brazil. This area suffered an environmental crime in 2015 when a rupture of an ore dam adversely affected water, soil, and air quality. Even today, Mariana and other nearby cities affected by this man-caused disaster have poor sanitation conditions and contaminated mudflow.

Colombo et al., (2018) characterized airborne VP isolated from two dairies in Italy. The authors raised some considerations about the safety of cheese ripening cellars, as human viruses belonging to the *Papillomaviridae* family showed a high abundance (17%) and identity higher than 90% with human papillomavirus. Although it is normally transmitted through direct skin-to-skin contact, the authors showed that even a slight human presence is sufficient for contamination, due to papillomavirus's high stability

outside the host. Proper industrial environment and hand workers sanitization are essential to avoid plant contamination. Although fermented foods pass through manufacturing processes that can inactivate viruses, many of them still carry infectious particles in the final product. The rapid identification of pathogens and monitoring sources of contamination are extremely important, especially for traditional fermented foods and minimally processed products (Maske et al., 2020).

RV and HEV are members of the enteric viruses' group that have zoonotic patterns (Leblanc et al., 2019). RV is distributed into ten groups or species (A to J) (International Committee on Taxonomy of Viruses, 2020) and are the most frequent species in human outbreaks. RV has segmented double-stranded RNA, which favors reassortments among human and zoonotic species. RV enteritis is frequently reported in calves and piglets in livestock (Martella et al., 2010). Contamination of food by RV occurs by primary source (water or infected animals) or along the food chain by food handlers. The WHO has recently estimated that 20 HEV infections occur annually worldwide (World Health Organization, 2020). HEV is a single-stranded RNA virus with four genotypes causing diseases in humans. Genotypes 1 and 2 infect only humans, mainly in undeveloped countries by contaminated water. Genotypes 3 and 4 have zoonotic patterns (infecting humans and other animals, mainly domestic pigs), and are associated with disease in industrialized countries after consumption of raw or undercooked meat from viremic animals. Genotype 3 is responsible for most zoonotic episodes. Normally, HEV causes self-limited disease, but in chronic liver disease patients and pregnant women may occur fulminant hepatic failure. HEV has been detected in meat, liver, kidney, and heart, principally from domestic pigs and wild boars (Doceul et al., 2016).

SARS-CoV-2

Severe acute respiratory syndrome Coronavirus 2 (SARS-CoV-2) is the causative agent of the ongoing coronavirus disease pandemic, named Coronavirus Disease 2019 (COVID-19) by WHO (Sohrabi et al., 2020). The SARS-CoV-2 is primarily transmitted by human-to-human transference via droplets (Lai et al., 2020). Other transmission routes include direct contact with an infected person and indirect contact through hand-mediated viral transfer from contaminated fomites to the nose, eyes, and mouth (La Rosa et al., 2020). Although there is no evidence that SARS-CoV-2 can be transmitted by food and water ingestion, the lack of scientific evidence leads to public health concerns mainly

because the virus can remain viable for days in favorable atmospheric conditions (Singhal, 2020).

Animal-to-human SARS-CoV transmission emerged through ingestion of Chinese ferret badgers, raccoon dogs, and Himalayan palm civets sold as food (Goli, 2020), representing the primary route of transmission. The most relevant animal reservoir of human MERS-CoV, for example, are dromedary camels that caused human–human infections as occurred in Saudi Arabia in 2012 (Park et al., 2018). Reusken et al., (2014) hypothesized a foodborne transmission through consumption of raw camel milk or raw meat, as antibodies of MERS-CoV were detected in serum and milk of dromedary camel. It was demonstrated that MERS-CoV experimentally introduced in camel milk can survive for up to 72 h at 4 ◦C and 22 ◦C (van Doremalen et al., 2014). Human Coronavirus (HCoV) was also experimentally recovered from lettuce after 4 days at 4 ◦C with titer 1.2 $\times 10^6$ (Yépiz-Gómez et al., 2013). The threat of secondary HCoV transmission through water used for food production is also hypothesized via aerosolization/fecal-oral route (Singhal, 2020).

Usually, thermal treatment at 60 ◦C for 30 min is sufficient to reduce SARS-CoV in free cell matrices (Goli, 2020). CoVs are also sensitive to basic and acidic pHs (Rabenau et al., 2005), but seem to be stable at 4 ◦C. It was demonstrated that viral infectious level declines faster at ~24 ◦C than at 4 ◦C (La Rosa et al., 2020). In addition, CoVs seem to be susceptible to chemical agents (e.g., salt and nitrates) and physical treatments (Thippareddi et al., 2020). Fermented foods are non-thermally or chemically treated and constitute a potential route of virus transmission. On the other hand, in pH around 5, SARS-CoV nucleocapsid starts to unfold and is denatured at a pH 2.7, suggesting the sensibility of SARS-CoV to pH changes (Wang et al., 2004). Innumerous fermented foods reach low pH levels and, consequently, can play a fundamental role in the elimination of the virus. All of these questions are hypothetical and should be the subject to study for verification.

After production, contamination still can occur at processing and during handling and delivery of food products by infected personnel via respiratory droplets, aerosols, or from contaminated equipment (Thippareddi et al., 2020). Recent SARS-CoV-2 outbreaks in food processing and food stores are highlighting the potential for employees being a source of contamination (Rizou et al., 2020). Considering the persistence of CoV on fresh food at 4 ◦C, the survival of SARS-CoV-2 on food packages was evaluated (Malenovská,

2020). The loss of infectivity of the virus on plastic surfaces was, on average, 0.93 log10 (i.e. 83%) per day of storage at 4 ◦C. However, when using wipes saturated with a combination of disinfectant agents (hydrogen peroxide and didecyl-dimethyl-ammonium chloride), it decreased the viral titter still more efficiently, by 3.8 log10 (99.98%). The Centre for Disease Control and Prevention (CDC) from the United States of America states that COVID-19 infection from handling contaminated food packages have low-risk, however, it is recommended cleaning and disinfection (Seymour et al., 2020).

Current virus detection methods

The recovery of viruses from fermented foods consists of their separation from other microorganisms and suspended solids (Barrangou et al., 2002). In liquid samples, the bacteriophage's separation process can be performed by ultracentrifugation, followed by a filtering step to eliminate contaminants. Solid samples must be previously added in a buffer or sterile culture medium and stirred to elute the phages from the food matrix (Foschino et al., 2005). If the sample contains a low phage titter, concentration by ultracentrifugation should be performed previously. For phage propagation, the filtrate is inoculated into a culture medium containing the host cell (Bandara et al., 2012; Lu et al., 2003a). After overnight incubation, the culture is ultracentrifuged, and the supernatant is filtered to remove the remaining bacteria cells. Then, the presence of phages and host range can be established by spot testing, plaque testing, or culture lysis. For classification, the most used method is the direct observation of morphology through Transmission Electron Microscopy (TEM), which despite being old, is still widely used. To a lesser extent, the PCR-based methods (e.g. RAPD, Multiplex PCR, MLST, and qPCR) and DNA analysis (DNA sequencing and RFLP) are eventually performed (Samson and Moineau, 2013b).

Pathogenic viruses, differently from bacteriophages, are rarely propagated in cell-culture assays, and its presence is based mainly on genome copy detection. Due to the significance of viral food borne diseases, validated methods for viral analysis in food are increasing over the years, such as the international standard method ISO 15216–1:2017 for NoV and HAV detection. Surrogates' viruses are frequently used on pathogenic viruses' trials. Surrogate viruses share molecular characteristics with pathogenic viruses, such as size and chemical composition (Richards, 2012). Murine Norovirus-1, Feline Calicivirus, and some human virus strains, adapted to cell culture propagation (HAV strain HM-175, Human Adenovirus, RV, Enterovirus, and others), are the most used in

cell culture-based assay or molecular techniques coupled to cell culture (Plaque Assay, Tissue Culture Infectious Dose (TCID) and Integrated Cell Culture Quantitative PCR (ICC-et-RT-qPCR)) (Cromeans et al., 2008). However, these trials are still not available in the routine of food analysis laboratories and the use of surrogate viruses is not fully elucidated to predict pathogenic viruses (Richards, 2012).

Pathogenic viruses were found in fermented food samples through PCR techniques [Quantitative (qPCR) and reverse transcription (RTPCR), or the combination of both (RT-qPCR)] (Table 2). They are prominent in sensitivity and specificity, being more employed than antigen detection and serology. In the PCR assay, DNA/RNA isolated from the target virus are amplified with specific primers. In the supplemental material, we compiled an extensive list of specific primers designed to detect viral pathogens (Table S1). However, PCR does not show virus viability and, to generate selectively amplifiable primers, the target sequence is required in advance (Sekse et al., 2017). This limitation is aggravated by the fact that novel viruses can arise, and the symptoms of viral diseases are very similar, making it difficult to know which virus is involved. To overcome this, Lee et al., (2018) proposed a PCR multiplex reverse transcription using six primer sets to simultaneously detect and quantify NoV, HAV, RV, and astrovirus in food samples (lettuce, oysters, and vegetable products). Considering the barriers, fast and more generalized techniques for identifying pathogenic viruses are required.

Table 2. Human pathogenic viral outbreaks associated with fermented food ingestion.

Virus	Classification	Fermented food	Location	Identification method	Disorder	Reference
TBEV	*Flavivirus*	Goat cheese	Germany	RT-qPCR	Meningitis, meningoencephalitis, or meningoencephalomyelitis	(Brockmann et al., 2018)
TBEV	*Flavivirus*	Goat cheese	Austria	Sample no longer available	Meningitis, meningoencephalitis, or meningoencephalomyelitis	(Holzmann et al., 2009)
TBEV	*Flavivirus*	Goat cheese	Croatia	Sample no longer available, however goats tested positive	Meningitis, meningoencephalitis, or meningoencephalomyelitis	(Markovinović et al., 2016)

VACV	*Orthopoxvirus*	Minas cheese	Brazil	qPCR	Skin lesions	(de Oliveira et al., 2018)
HEV	*Orthohepevirus*	*Figatelli*	Southeastern	RT-PCR	Acute and chronic hepatitis, death	(Colson et al., 2010)
HEV	*Orthohepevirus*	Raw pork sausage	Netherlands	RT-qPCR	Acute and chronic hepatitis	(Boxman et al., 2020)
NoV	*Calciviridae*	Fermented oyster	South Korea	RT-PCR	Acute gastroenteritis	(Cho et al., 2016)
NoV	*Calciviridae*	Kimchi	South Korea	RT-PCR	Acute gastroenteritis	(Park et al., 2015)
RVA	*Rotavirus*	*Minas frescal* cheese	Brazil	qPCR	Acute gastroenteritis	(de Castro Carvalho et al., 2020)
NiV	*Henipavirus*	*Tari* (fermented palm sap liquor)	Bangladesh	Sample no longer available	Encephalitis and death	(Hossain et al., 2016)

Tick-borne encephalitis virus: TBEV; Vaccinia virus: VACV; Hepatitis E Virus: HEV; Norovirus: NoV; Rotavirus A: RVA; Bat Nipah virus: NiV.

Food virome

Great effort over the last years has been made to prevent virus contamination during fermentation processes (Park et al., 2011). However, surprisingly, viral presence started to be more noticed with the advancement of NGS technologies, and it was reframed in the fermented food niche. This transition was decelerated because food microbiome investigations are mainly focused on the characterization of bacterial and fungal communities, viruses being largely neglected (Ledormand et al., 2020).

The challenges of using NGS to characterize viral content are numerous, including the absence of universal marker genes (Eric Wommack et al., 2012), the low concentration of viral DNA for preparation of genomic libraries (Garmaeva et al., 2019), the contamination of the sample with bacterial and fungal DNA (Kim and Bae, 2011), and the scarcity of databases for virus sequences as they remain largely unknown (Bikel et al., 2015; Garmaeva et al., 2019). Nevertheless, some food microbiome studies have detected, but not classified viruses in fermented food systems (Liu et al., 2020; Lyu et al., 2013). To a lesser extent, some studies have achieved classification, even though it was focused on bacterial and fungal communities (Agyirifo et al., 2019; Illeghems et al., 2012; Kumar et al., 2019).

Kumar et al., (2019) analyzed kinema (traditional fermented soybean from Himalaya) samples using Illumina NGS technology and found less than 1% of total reads

belonging to bacteriophages, with *Siphoviridae* family being dominant. Illeghems et al., (2012) and Agyirifo et al., (2019) analyzed cocoa beans by Illumina and reported 0,25 and 1%, respectively, of the total metagenomics read sequences being bacteriophages. Both studies reported *Lactobacillus* phages of *Siphoviridae* family as the dominant, and the presence of phages infecting *Enterobacter, Klebsiella, Pseudomonas, Bacillus,* and *Staphylococcus*. Interestingly, Agyirifo et al. (2019) attributed *Lactobacillus* bacteriophages to positively influence the aroma formation during cocoa beans fermentation. The authors mentioned that bacterial cell lysis caused by phages releases intracellular enzymes in the food matrix, degrading the substrate, and stimulating aroma production.

Currently, there are two approaches for metagenomic studies; i) metagenomic amplification of the target gene, where only a specific region is sequenced, performed to characterize mainly bacterial and fungal communities, and ii) shotgun metagenomics which consists of the sequencing of random fragments of all microbial DNA present in a given sample, frequently used in viral communities' studies (Sharpton, 2014). In any case, due to these advances, the terms "ome" and "omics" have been attributed to viruses.

The "virome" and the (meta) viromics refer to all viruses present in each sample and the study of their genomes, respectively (Garmaeva et al., 2019). However, since most existing phages belong to the *Caudovirales* order, current studies on viral communities in fermented foods are strictly focused on the presence of dsDNA phages, unfortunately excluding the possibility of identifying other viruses and RNA-containing phages (Mokili et al., 2012). Most pathogenic viruses associated with fermented foods, due to their RNA genetic material content, cannot be identified by next-generation approaches. However, when associated with PCR, it seems to be a promising tool for food safety monitoring. Metabarcoding strategy after RT-qPCR sample treatment has successfully been used, despite being limited to closely related viruses or viral families (Desdouits et al., 2020). If food is contaminated by innumerous viral strains belonging to different genotypes, PCR products are synthesized for each strain, and bioinformatics analyses each reading and classifies genotypes. Imamura et al., (2017) used PCR primers targeting the N-terminal area of the VP1 protein and analyzed the diversity of NoV genogroups I and II in naturally contaminated oysters with Illumina platform. Oshiki et al., (2018) combined a micro fluidic tool allowing the use of multiplex PCR and Miseq sequencing to the detection of 11 different human RNA viruses from human feces,

sewage, and oysters artificially contaminated. Even so, these techniques require more studies to attend to fermented foods, whereas they have peculiar characteristics affecting viral content access.

Alternatively, to the term “virome”, studies of the viral community are called frequently by “phageome” (Ledormand et al., 2020). Figure. 2 illustrates the NGS standard workflow for viral community analysis in fermented foods, including (1) sampling, (2) DNA extraction, (3) library preparation, (4) sequencing, (5) data analysis, and (6) results.

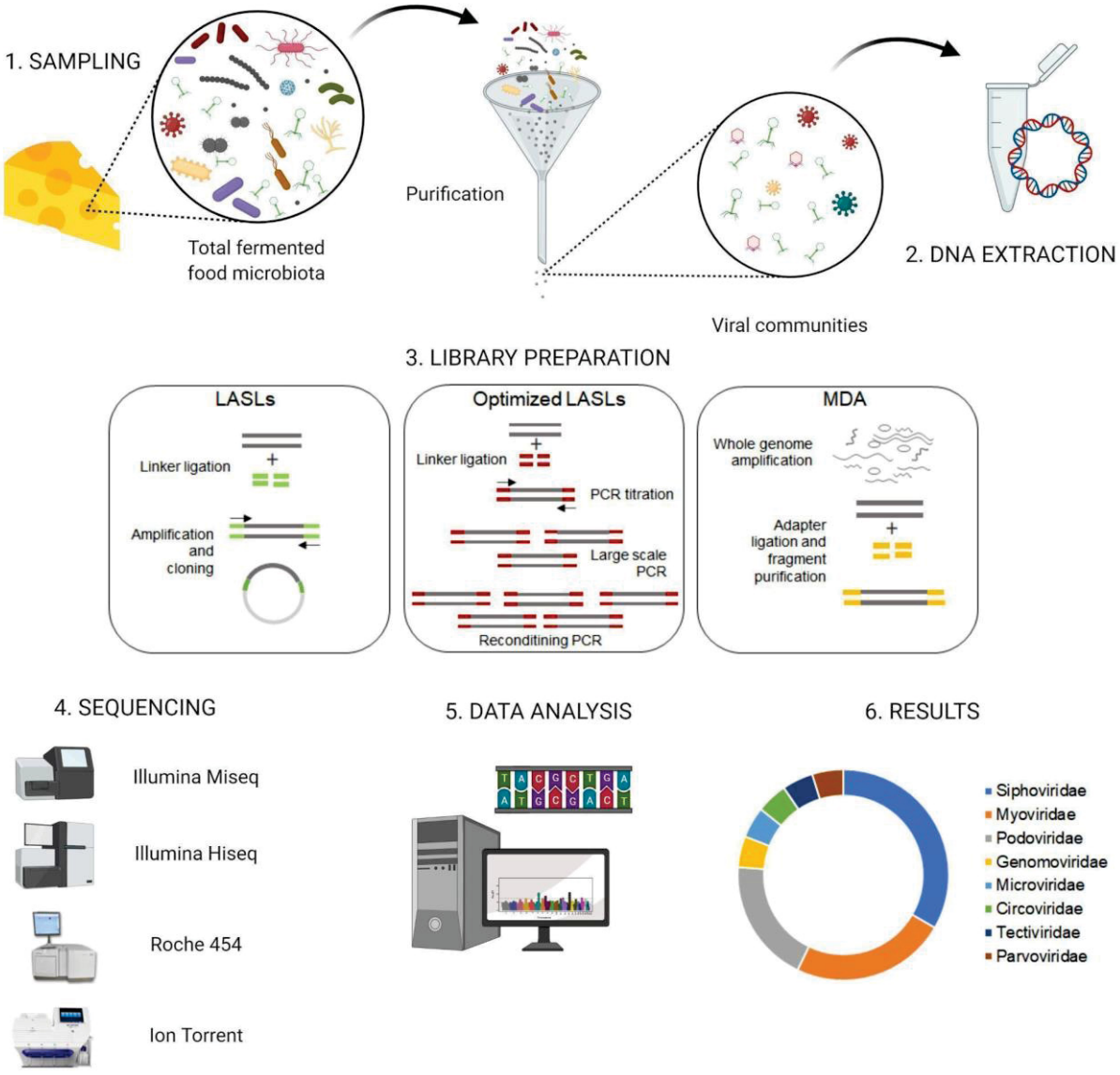


Figure. 2. Schematic workflow for analyzing virus communities in fermented foods by next-generation sequencing. LASLs (linker amplification shotgun libraries) and MDA (multiple displacement amplification). This figure was created using BioRender (https://biorender.com/).

Sampling and DNA extraction

All virome study starts with the step of sample purification. The purification method must be able to satisfactorily represent the original virus population (Hayes et al., 2017). Usually, the purification of the viral particles (VPs) is divided into three steps: i) VPs recovery, ii) VPs purification and concentration, and iii) an optional second purification via cesium chloride gradient (Park et al., 2011). Although there is no standardized sampling protocol for all fermented foods, recently, Dugat-Bony et al., (2020) optimized a method for extraction and purification of bacteriophages from feces to perform, for the first time, viral metagenomic analysis of cheese surface. They used a chloroform treatment and filtration steps to extract viral DNA prior to the Illumina Miseq sequencing.

Virome studies of other fermented foods (e.g., kimchi, sauerkraut, and fermented shrimp) have used different strategies, mainly in the VPs concentration step. While Park et al., (2011) used ultracentrifuged at 100,000×g for 4 h at 4◦C, Jung et al., (2018) opted to precipitate the VPs with polyethylene glycol 8000 e NaCl 1 M. In addition, due to the high concentration of cellular microorganisms in fermented food, samples are prone to contamination by bacteria. An alternative is the treatment of concentrated VPs with lysozyme and chloroform, followed by incubation with DNase and RNase to remove the remaining genetic material (Dugat-Bony et al., 2020; Jung et al., 2018; Park et al., 2011). Regardless of which purification technique is employed, a step of eliminating bacteria and fungi also means eliminating prophages that are frequently inserted in the genome of these hosts, generating a bias for the technique, as it considers only the free phage (Sausset et al., 2020).

Nevertheless, subsequently, the extraction of DNA from the VPs is performed with commercial kits. It is important to note that the choice of extraction kit influences the composition of the microbial community produced by NGS and may generate inaccurate results. Therefore, the extraction method should be chosen according to the objective of the researcher. Also, it is recommended that after viral DNA extraction a small aliquot should be amplified by PCR using 16S/18S rRNA primers to ensure the absence of bacteria and fungi DNA contamination (Hurwitz et al., 2016).

Library preparation and sequencing

Most of the viromes that have been performed so far have used linker amplification shotgun libraries (LASLs) or whole (meta) genome amplification (WGA) methods [e.g., multiple displacement amplification (MDA)] (Willner and Hugenholtz, 2013). LASLs consist of the random fragmentation of genomic DNA and the binding of known linkers in these fragments that can be used for PCR amplification. At the time this method was developed, the fragments were cloned in plasmid vectors and sequenced by the Sanger method to generate viral metagenomes. However, this strategy had the bias of large-scale cloning and sequencing (Breitbart et al., 2002).

NGS technologies eliminated the need for cloning vectors and significantly increased the sequencing capacity; however, the low viral DNA concentration remains a challenge since some NGS protocols require nucleic acid micrograms (Duhaime et al., 2012) optimized the LASLs technique with the addition of a titration step, significantly reducing the number of cycles and the DNA concentration required to build the library (only 1 pg), enabling large scale PCR (Willner and Hugenholtz, 2013). The optimized linker amplification (LA) technique has been adapted for 454 sequencing but also can be used to build genomic libraries on other sequencing platforms, such as Illumina and Ion Torrent (Duhaime et al., 2012).

The MDA method uses the high efficiency of polymerase ϕ29 which synthesizes >70,000 nucleotides per cycle from small concentrations of DNA, allowing the amplification of complete viral genomes through adapter ligations followed by purification step (Bikel et al., 2015). LA and MDA are powerful tools for studying virome, however, MDA is prone to unevenly amplify linear genome fragments and might generate biases into the representation of ssDNA circular viruses (Kim and Bae, 2011), while A-LA provides a more reliable representation of ssDNA and dsDNA viruses (Roux et al., 2016).

After the construction of the genomic libraries, the sequencing is performed using mainly the Illumina, Roche 454, and Ion Torrent platforms (Hayes et al., 2017). The quality control steps of the generated sequences must be performed as reviewed by Hurwitz et al. (2016). Succinctly, quality control consists of ensuring optimal sequencing coverage for each sample and removing rare reads that may occur from sequencing errors

or contamination of the sample, leading to over estimation of the virome diversity (Hurwitz et al., 2016).

Data analysis

The central challenge in bioinformatics analysis of viromes is the absence of universal genes, which makes diversity estimation difficult. Currently, taxonomic classification is often performed by aligning the generated sequences against a "generalist" database using the BLAST (Basic Local Alignment Search Tool) or BLAST-based programs, such as MetaPhyler (B. Liu et al., 2011), CARMA (Gerlach et al., 2009), and MG-RAST (MetaGenomic-Rapid Annotation using Subsystem Technology) (Glass et al., 2010). However, a large part (about 60–99%) of the sequences produced in viromes has no homology with viral sequences available in the databases.

To solve this problem, database focused on the taxonomic classification of viruses [ACLAME (Leplae et al., 2009) and Phage SEED (Overbeek et al., 2005)] and specific data analysis programs [VIROME (Viral Informatics Resource for Metagenome Exploration) (Eric Wommack et al., 2012), VMGAP (Viral MetaGenome Annotation Pipeline) (Lorenzi et al., 2011), and Metavir (Roux et al., 2014)], were developed. Usually, these pipelines use an ORF (open reading frame) localization algorithm and perform a comparison with a protein database (Hayes et al., 2017) creating a functional and taxonomic profile of the viral community (Bikel et al., 2015). In addition, an independent method of similarity PHACCS (PHAge Communities from Contig Spectrum) software was developed to better understand the structure of the viral community. It provides the estimation of diversity and uniformity revealing the most abundant viruses in a viral metagenome. This analysis is based on the principle that the most abundant virotypes in a VPs sample will more likely be assembled into large contigs (Reyes et al., 2012).

Results

The diversity revealed by virome studies in fermented foods agrees with previous findings described by culture-dependent methods. Most of the sequences belong to Caudovirales order of the families *Siphoviridae*, *Myoviridae*, and *Podoviridae* (Agyirifo et al., 2019; J. H. Cheng et al., 2018; Del Rio et al., 2019; Illeghems et al., 2012; Jung et

al., 2018; Kot et al., 2014; Kumar et al., 2019; Park et al., 2011). Similar results are seen on the human gut virome (Garmaeva et al., 2019).

Park et al. (2011) applied pyrosequencing on fermented sauerkraut, kimchi, and shrimp samples and verified that *Siphoviridae* dominated sauerkraut (60.07%) and fermented shrimp (53.55%), differently from kimchi, which *Podoviridae* prevailed (52.82%). They also first identified *Phyconaviridae* in a fermented food, an attacker of harmful eukaryotic algae responsible for algal blooms, demonstrating that metagenomics studies contribute to the discovery of biocontrol agents for different fields other than food. Jung et al. (2018) described the dominance of ssDNA viruses in Korean kimchi, including *Circoviridae* (hosts: birds and pigs), *Genomoviridae* (plants and fungi) and *Microviridae* (bacteria), not previously described. They applied Illumina HiSeq to differentiate Chinese and Korean kimchi origins and found that viral clusters were more clearly distinguished than bacterial clusters by beta diversity analysis, making viruses more strongly associated with the geographic origins of fermented foods than the bacterial ones. The origin of traditional food reveals its quality and safety, being of great importance to consumers.

Beneficial viruses: the other side of the coin

Phages directly control bacterial dynamics, promoting their balance in fermentation. In the ecological study of sauerkraut fermentation, (Lu et al., 2003b) observed that the succession of bacteria was associated with the content of the respective phages found in different stages of fermentation. At the beginning of the process, *Leuconostoc* spp. and *Weissella* spp. prevailed and the phages that infected these species were isolated. After seven days, *Lactobacillus* was the main genus, and phages infecting them were observed. Recently, Kumar et al. (2019) also noticed that phages seem to determine the abundance of bacterial communities during the fermentation of Kinema, acting in the biocontrol of *Bacillus, Staphylococcus, Enterococcus, Lactococcus,* and *Streptococcus.*

These results suggest that phage dynamics during fermentation is crucial to guarantee a good succession of bacteria. In addition, phages can move between different environments, elevating horizontal gene transfer (HGT) and, therefore, forcing bacteria to evolve (Breitbart and Rohwer, 2005). Understanding the process of phage-bacteria

evolution assists the selection of good strategies to control harmful phages and maintain beneficial ones, improving fermentation performance.

Phages can act as biocontrol agents combating pathogenic and deteriorating bacteria, as well as toxic metabolic components in fermented foods (García et al., 2008). Bandara et al., (2012) found two phages belonging to *Myoviridae* family were capable of eradicating *Bacillus cereus* quickly when supplemented with divalent cations (Ca^{2+}, Mg^{2+} or Mn^{2+}) in cheonggukjang, a product of fermented soybean mass. Philippe et al., (2018) found a phage that infects *Gluconobacter cerinus*, a spoilage acetic acid bacterium in the wine making process. *G. cerinus* produces ethyl alcohol and transforms it into acetic acid, representing hazardous to the final product. Additionally, phages can also combat toxic metabolic components released by LAB in fermented foods (Del Rio et al., 2019; Ladero et al., 2016). Recently, Del Rio et al. (2019) proved the efficiency of an *Enterococcus faecalis* bacteriophage of *Myoviridae* family in reducing biological amines (BA) in an experimental model of cheese. Phage presence reduced BA and putrescine without devastating the starter culture due to its specificity for *E. faecalis*.

The discovery of other perspectives for phages that infect LAB was also enabled. Phages that attack *Streptococcus thermophilus* were associated for decades as the cause of defects and flaws in the yogurt fermentation process (Bendadis et al., 1990; Brussow et al., 1994; Ishlimova et al., 2012; Ma et al., 2014; Quiberoni et al., 2003). However, recently, Pacini and Ruggiero, (2019) attributed a probiotic potential to fermented milk and colostrum after analyzing the genomes of innumerous *Streptococcus* and *Lactococcus'*s phages contained in the product using Axiom Microbiome Array. When ingested with food, phages can influence the host in three ways: i) modulation of the gastrointestinal microbiota, as they can act against pathogens and, by promoting horizontal transfer of genetic material, it operates in the improvement and evolution of bacterial community diversity; ii) intestinal mucosa cell interaction, indirectly triggering immune system response; and iii) immune system components interaction, directly driving immune response, as they can overcome anatomical and physiological barriers, being found in compartments of the human body earlier considered sterile (Sausset et al., 2020). In this way, *Lactococcus* and *Streptococcus* phages can possess antimicrobial, antitumor, and antiviral effects on the host. In addition, phages may interact with the immune system, opening the possibility to immunotherapy to treat diseases such as cancer and autism. Phages presence can enhance notable benefits, which are still untapped.

Final considerations

The worldwide emergence of COVID-19 resulted in abrupt awareness of the presence of viruses in all sectors of the economy. Cases of NoVs, RVs, and HV have been reported in association with cheeses, sausages, fermented vegetables, and fermented cereals. However, there is no evidence of COVID-19 transmission through fermented foods. The possibility of food transmission needs to be investigated and clarified since no food virome study has been conducted since the first case identified in China, in December 2019. Studies have reported that Coronaviruses can remain infectious in waters and are highly stable at 4 ◦C, the main raw material, and storage temperature, respectively, of fermented foods. While respiratory droplets are the main way the virus spreads, transmission via fermented foods is considered negligible. However, transmission appears to be possible if the virus is transferred from hands to food and the food itself to the mucous membranes of the mouth, throat, or eyes.

A recent study showed that physical contact and shared food during a conference in Singapore resulted in a cluster of COVID-19 patients (Pung et al., 2020). The major risk enhancing factors of fermented foods is the use of contaminated raw materials, the conduction of poorly controlled natural fermentation, and the lack of pasteurization. Thus, to minimize the risk of virus contagion, good hygiene practices and cleaning of the fermentation room (taps, door handles, fermentation vessels, and utensils) should be followed by hand washing or using hand sanitizer.

On the other side of the coin, emerging evidence of bacteriophage diversity in fermented foods by NGS has revealed an ongoing paradigm in understanding their role in this ecosystem. The positive influence of phage is ample, ranging from sensorial improvement of the fermentation process to probiotic potential by cell interaction. To overcome unexplored fermented food virome ecosystems, current challenges, such as the expansion of databases for non-cultivable viruses, the optimization of isolation protocols, and new bioinformatics tools, demands to be faced. Establishing viromes of innumerous fermented foods, studying the long-term evolution of virus-bacterial interactions, analyzing the influence of external factors, and elucidating the interaction of viruses with consumer’s gastrointestinal tract cells will be essential in keeping viruses as allies.

CHAPTER FIVE - YEAST VIRUSES AND THEIR IMPLICATIONS IN FERMENTED FOODS AND BEVERAGES

Manuscript published in *Current Opinion in Food Science* journal. DOI: https://doi.org/10.1016/j.cofs.2022.100879

Abstract

The discovery that yeasts are natural hosts for viruses in the 70s marked the beginning of research into yeast virology and, particularly, the killer yeast system. These viral sequences encode host toxin secretion, modulating microbial communities, and ecosystem functions in fermentation processes. Wine is the most frequently studied fermented food for this topic, and the presence of dsRNA virus of the family *Totiviridae*, infecting mainly *Saccharomyces* spp., was initially associated with fermentation failure. After being better described, the possibility of the beneficial use of the 'Killer yeast' was raised, and food technologists started to develop robust strains to modulate final product quality. In addition, with the advancement of next-generation sequencing and food virome studies, new viral groups and beneficial ecological functions have been revealed. This review addresses important topics on yeast virus and fermented foods, including diversity, ecology and applications, and a patent landscape and detection methods of the killer yeast system.

Introduction

Viruses are widespread infecting all domains of life. Exploiting the living creatures on the planet, they present high reproduction capacity and high genetic variability, adapting faster than any species. Fungal viruses, called mycovirus, are widespread in the Fungi domain and are usually associated with symptomless infections (Ghabrial, 1998). In the current scientific literature, viromes are dominated by phages in food microbial ecosystems, as most fermentations harbor bacteria (Maske et al., 2021a). So far, no study has characterized the eukaryotic virome diversity of complex fermented food samples. Few studies have dealt with this topic, describing yeast virus on selected yeast strains (Table 1). Fermented foods contain more simplified viral communities than other samples from human feces, soil, and marine sediment (Jung et al., 2011). Fungi, especially yeasts, play a significant role in food fermentation, such as wines, beers, bread, cocoa, and coffee (Mannaa et al., 2021). Fungal viruses are mainly double-stranded RNA (dsRNA) or positive single-stranded RNA (+ssRNA), and some have linear negative (-ssRNA) and circular ssDNA genomes (Ghabrial et al., 2015). dsRNA mycoviruses are arranged in eleven families and +ssRNA genomes into eight families listed by Ghabrial et al., (2015). The best-known dsRNA mycoviruses of the family *Totiviridae* infects *Saccharomyces cerevisiae*, the most widely spread yeast employed in fermented foods (Maicas, 2020).

Even though most of the mycoviruses appear to be harmless, they can cause phenotypic changes in their hosts (e.g.: pigmentation abnormalities; growth rate; sporulation taxa; stress tolerance; hypo- or hypervirulence; enable the production of extracellular antifungal toxins) (Jagdale and Joshi, 2018). Toxins are encoded by yeast viral ‘killer system’. Among mycoviruses, killer system allows yeast to produce extracellular toxins, which is composed of two cytoplasmic dsRNA viruses: the cytoplasmically inherited toxin-encoding M-dsRNA satellite virus, and the L-A helper virus, that assists replication on host cell (Schmitt and Breinig, 2002). The presence of the viral sequence can lead to an intraspecific interference competition during the fermentation process. The killer yeast biocontrol action was suggested for beer production by Kordialik-Bogacka, (2022). Zymocins are polypeptides produced by killer yeast species and can be used as antimicrobial metabolites against undesirable deteriorating strains. Since viral ecological interactions can shape the fermentation course, plenty of possibilities can be raised and explored to improve the quality and safety of the

fermentation process (Jagdale and Joshi, 2018). In this way, much effort has been done to determine protocols for fully elucidating virome taxes. Most yeast viruses have linear dsRNA genomes, hampering the use of their nucleotide sequence by conventional sequencing methods (Crabtree et al., 2019). Rapid extraction and high-quality cDNAs for downstream next-generation sequencing (NGS) began to be unraveled (Crabtree et al., 2019). This overview presents the advances related to the diversity and application of the eukaryotic virus in applied food microbiology.

Table 1. Yeast virus groups diversity involved in food fermentation.

Family/genus	Yeast species (host)	Interest/ source	Genetic configuration	Identification method	Virus location	Reference
Totiviridae	*T. delbrueckii*	Wine	dsRNA	HTS	Cytoplasm	(Jackson, 2008)
Totiviridae	*S. cerevisiae*	Wine	dsRNA	HTS	Cytoplasm	(Jackson, 2008)
Totiviridae	*T. delbrueckii*	Antifungal activity (wine)	dsRNA	HTS	Cytoplasm	(Gier et al., 2020)
Totiviridae	*S. cerevisiae* 2928	Wine	dsRNA	*PCR*	-	(Lee et al., 2022)
Totiviridae	*S. cerevisiae*	Grape must fermentation	dsRNA	*PCR*	-	(Ramírez et al., 2022)
Totiviridae	*S. cerevisiae*	Wine/Antifungal activity	dsRNA	*RT-PCR*	Cytoplasm	(Ramírez and Antonio, 2021)
Totiviridae	*S. bayanus*	Grapes/Pulp	dsRNA plasmid	PCR	Cryptic plasmid	(Rodríguez-Cousiño et al., 2013)
-	*S. bayanus var. uvarum*	Wine	dsRNA	Gel electrophoresis	Cryptic plasmid	(Velázquez et al., 2015)
Partitiviridae (Cryspovirus)	*S. cerevisiae*	Grape must/Beer	dsRNA	HTS	-	(Mannaa et al., 2021)

Totivirus	*S. cerevisiae*	Grape must	dsRNA /satellite	HTS	*Cytoplasm*	(Mannaa et al., 2021)
Narnavirus	*S. cerevisiae*	Grape must	*ssRNA*	HTS	-	(Mannaa et al., 2021)
Totivirus	*S. bacillaris*	Grape must	dsRNA	HTS	-	(Mannaa et al., 2021)
Mitoviridae	*S. bacillaris*	Grape must	*ssRNA*	HTS	-	(Mannaa et al., 2021)
Narnavirus	*S. cerevisiae* I-329	Industrial production of sherry-like wines	*ssRNA*	RNA-Seq	-	(Rodríguez-Cousiño et al., 2011a)
Totiviridae	*S. paradoxus* AML-15-66	Spontaneous fermentation of serviceberries	dsRNA	PCR	Telomeric region of chromosome 5	(Naumov et al., 2009)
-	*S. bayanus*	Grapes/Wine/ Must	dsRNA	PCR	-	(Ivannikova et al., 2007)
Totivirus	*P. membranifaciens*	Draught beer	dsRNA	HTS and RT-PCR	-	(González-Alonso et al., 2021)
Totivirus	*S. cerevisiae*	Wine fermentation	dsRNA	Karyotype analysis (PFGE)	-	(Mardanov et al., 2020)

HTS: High-throughput sequencing; RT-PCR: Real-time PCR; PFGE: Pulsed field gel electrophoresis.

Yeast viruses in fermented food

The majority of mycoviruses genomes consists of dsRNA and 30% is composed of a +ssRNA (Son et al., 2015). They are in single or mixed infections in both laboratory and wild strains. The first yeast virus was revealed by Bevan et al., (1973), who reported two dsRNA and proposed their relationship to the killer character in *S. cerevisiae*. Subsequently, Field et al., (1982) explored differences between *S. cereviseae* L-A helper virus. The killer system virus inhabits cytoplasm; however, other mycoviruses as well as homologous ORFs of toxin-encoding dsRNA viruses can be inserted on plasmids or also in chromosomes (Figure 1) (Son et al., 2015). Killer phenotype depends on the secretion

of low molecular mass proteins (the killer toxins), which kill sensitive cells of the same or related yeast genera without direct cell-to-cell contact. The killer strains themselves are immune to their toxin but remain susceptible to the toxins secreted by other killer yeasts (Schmitt and Breinig, 2002). The toxins are divided in K1, K2, Klus and K28 according to their killing behavior and a shortage of cross-immunity (Matilde Maqueda et al., 2012). The toxins are divided in K1, K2, Klus and K28 according to their killing behavior and a shortage of cross-immunity (Matilde Maqueda et al., 2012). These toxins differ not only in molecular size but also in their mechanisms of action and cellular targets: K1 and K2 toxins primarily disrupt the plasma membrane by forming pores, leading to ion imbalance and cell death, whereas K28 enters the target cell and inhibits DNA synthesis by interfering with nuclear functions. Therefore, their metabolic impacts range from membrane destabilization to the arrest of cellular replication processes.

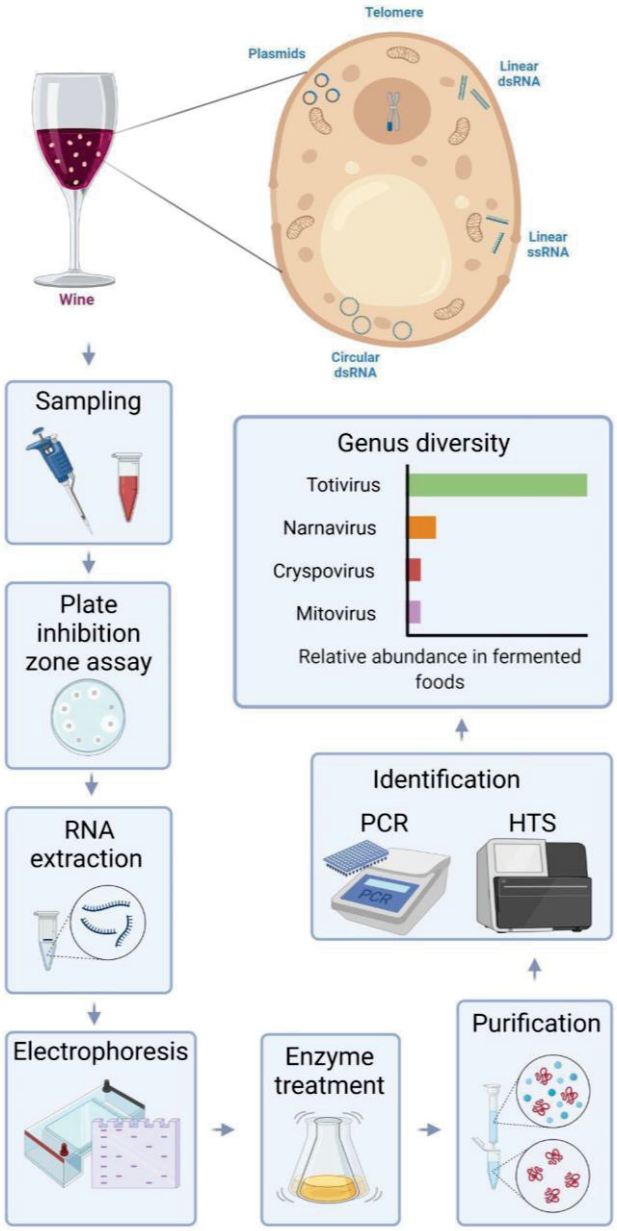


Figure 1. Representation of viral sequence's location in yeasts and detection methods of killer yeast species. Legend: (a) Diversity of mycoviral sequences in yeast host model; (b) Steps on selection of killer yeast strains: 1) sampling, 2) selection by plate inhibition zone essay using, for example, MB; 3) Genetic material extraction; 4) Electrophoresis to understand killer phenotype; 5) Enzyme treatment to digest genetic material and 6) purification to avoid contaminants; and 7) Sequencing through PCR or HTS (Illumina sequencer).

S. cerevisiae is widely used in the production of fermented food and beverages, being an object of foremost importance for fermentation processes (Maicas, 2020). The most characterized are wine yeast viruses, collected from grapes or must (Table 1). Although *S. cerevisiae* is the principal yeast, recent studies have been elucidating the impact of the variety of autochthonous species (e.g.: *Metschnikowia pulcherrima*;

Lachancea thermotolerans; Hanseniaspora uvarum; Torulaspora delbrueckii) carried out by grapes that affect oenological properties (González-Alonso et al., 2021). Mapping the diversity of viruses that are inoculated through these strains is crucial. *Totiviridae* is the most widespread family among the *Saccharomyces* yeasts and is traditionally represented by four L-A genotypes, three L-BC of *S. cerevisiae*, and four M dsRNAs satellite (Rodríguez-Cousiño et al., 2011a). The majority of commercial *S. cerevisiae* strains host helper L-A and satellite M dsRNAs linked to killer phenotype (Crucitti et al., 2022). The use of commercial strains with active killer systems, that also are immune to other killer strains, supports the industrial fermentation process's success (Jackson, 2008).

Mycoviruses are transmitted intracellularly during division because they lack an extracellular phase in their life cycles (Ghabrial et al., 2015). This lack of extracellular transmission route is attributed to the physical barrier of the fungal cell wall that generally does not allow direct viruses uptake. Instead, they are transmitted by vegetative cell division, asexual and sexual reproduction (sporogenesis process), or cell fusion (Son et al., 2015). Opposite to lytic viruses, they establish an endosymbiotic relation with the yeast host, boosting both reproductive successes (Lee et al., 2022). Their natural host ranges are limited to individuals within the same or closely related vegetative compatibility groups (Ghabrial, 1998), resulting in narrow coevolution. *Totiviridae* family viruses were reported recently in non-*Saccharomyces* species, *T. delbrueckii*, and *Pichia membranifaciens*, by high-throughput sequencing (HTS) methods (González-Alonso et al., 2021; Ramírez et al., 2022; Rodríguez-Cousiño et al., 2011b). Due to the advances of cultivation-in-dependent techniques in virome research, novel groups are continuously arising. The presence of *Partitiviridae* and *Mitoviridae* in yeasts of oenological interest was reported for the first time using HTS on RNA (Crucitti et al., 2022). Four novels putative mycoviruses across Totivirus, Cryspovirus, and Mitovirus genera were reported.

Ecology and evolution of killer system

Killer phenotype is markedly in multiple fungal taxonomic groups from different habitats. Especially in wine fermentation, their frequency is variable, and they can be up to 88% of spontaneous fermentation yeasts (M. Maqueda et al., 2012). The permanence

of viral sequences seems to be dependent on the abiotic environment. López et al., (2002) demonstrated that the copy number of Narnavirus increased in industrial and laboratory *S. cerevisiae* strains exposed to nutritional stress conditions, without any correlation between the viral infection and biological characteristics of the strains. Also, dsRNA, either killer or helper viruses, are occasionally lost after heat treatments in the laboratory, suggesting that they are susceptible to temperature fluctuation (Boynton et al., 2021). Environmental stress factors faced through industry processes can result in phenotypic changes in yeast such as sporulation taxa (Ravoitytė et al., 2020). Ravoitytė et al. (2020) evaluated, for the first time, the genome alterations of *S. paradoxus* on behalf of dsRNA viruses. The authors showed that viral presence was connected to innumerous cellular mechanisms regulating modification of gene transcription, which impacted the metabolism of lipids, amino acids, carbohydrates and nucleotides. In contrast, for a long time, mycovirus infections were usually associated with mild symptoms to the host. The toxin secretion increases the competitiveness of killer yeast toward non-killer strains in a shared habitat without direct cell-to-cell contact, resulting in multiple ecological interactions (Boynton, 2019; Lee et al., 2022). Toxins are a selective pressure that leads to an immune response. Sensitive strains create self-protection against exogenous killer toxins. Gier et al. (Gier et al., 2020) utilized two K1 killer strains with different toxin rates and sensitivities to K1 toxin. The authors evaluated cellular adaptations on lipidome, and transcriptome levels caused by intrinsic K1-induced pressure and found that K1 induces changes in gene expression in both short and prolonged incubation periods. Yeast ecology can be used in favor of food fermentation. However, its effectiveness is dependent on killer neighboring cells' sensitivities, distances, time of contact, and densities, as well as abiotic conditions such as pH, temperature, salt, and nutrient (Boynton, 2019).

The origin of mycoviruses is uncertain. It is hypothesized by phylogenetic analysis that the association between mycoviruses and their host is ancient and reflects a long period of coevolution (Son et al., 2015). In respect to the evolution of the killer system, it is possible that the encoding killer toxins system is linked to the yeast chromosome (Rodríguez-Cousiño et al., 2011a). Rodríguez-Cousiño et al., (2011b) raised, for the first time, the hypothesis of viral sequences originating from host chromosomal genes, after reporting homology between toxin-encoding M satellites (Klus toxin) and an ORF from yeast genome. These findings suggest an evolutionary correlation and, once independent from DNA replication, Klus might evolve at a faster pace than the

host. More recently, another evidence of homology was proposed by genetic sequencing of *S. paradoxus* viral toxin K1-like and K1 toxin from *S. cerevisiae* (Fredericks et al., 2021). Due to the low incidence of viruses in wild yeasts, some studies suggest a detrimental impact of the virus on yeast, probably due to the competition for energy and material resources from the host (Ravoitytė et al., 2020). Indeed, there is a host machinery effort to maintain viral sequence replication. On the other side, killer viruses provide competition benefits to yeast hosts (Pieczynska et al., 2017). More recently, Gier et al., (2020) suggested the correlation of the host cell's ability to deal with toxin molecules as an intrinsic selective pressure. Despite the effort, killer toxins can improve yeast fitness in a microbial community. A multilevel virus/host evolution case study was done to evaluate the adaptive evolution of killer yeasts and shows a resistance against killer viruses and mutations (Buskirk et al., 2020). Adaptation of yeast nuclear genome due to killer phenotype results in deterioration of viral sequences. In contrast, an experimental study done by Pieczynska et al., (2017) suggests a mutualistic symbiosis between killer virus encoding toxin type K1 and *S. cerevisiae*. Close coadaptation could be inferred as more recently virus–host pair had lower ability on killing sensitive strains than native virus–host pairs, i.e., when imposing stressful conditions, recent virus–host pair are more susceptible to lose connection than native ones; and host fitness increases when is introduced to native pairs and decreases when is inserted with new viral killer sequences (Pieczynska et al., 2017). Long-term coevolution was also suggested by Lukša et al., (2017). The authors identified that more than 700 genes can be affected by elimination of dsRNA viruses. Taking all together, a deeper investigation of the yeast-host interface is essential for understanding this pair's coevolution.

Effects on fermented foods and technological approaches

Yeasts are reservoirs of viruses and, once in fermented foods, can carry a diverse viral community to human gastrointestinal microbiota (Maske et al., 2021a). To date, there are no reports of yeast viruses posing a threat to human health. The killer toxin from *Tetrapisispora phaffi* Kpk1 expressed in *Komagataella phaffii*, possessing a biocontrol action against wine spoilage strains, was tested on cells from human keratinocyte HaCaT. The study showed that the dose used in food additives (0.25–2 AU/mL) had no cytotoxic effect (Carboni et al., 2021). However, yeasts are important eukaryotic model organisms to virus research (Sahaya Glingston et al., 2021). Some essential cellular processes are conserved from yeast to higher eukaryotes, and viruses target these conserved regions for

successful infection. There has been very limited investigation into the viral content diversity of fermented food communities. In this way, it is important to understand complex communities to predict potential impacts on human health.

Once the killer system was discovered, it was ascribed as a cause of fermentation flaws. The toxins secreted by wild yeasts inoculated from outside sources, like the grapes, in the case of wine, occasionally suppress commercial *S. cerevisiae*-sensitive yeast in the wine fermentation process (López et al., 2002). This reflects on the necessity to use commercial strains with an active immune system against killer yeasts or inactivate these contaminants in the early stage of the process. After being better described, the possibility of its beneficial use was raised. Food and beverage industries were the first to implement killer toxins as biocontrol agents to kill spoilage strains in beer, wine, and bread (de Ullivarri et al., 2014; Nouri Alturki et al., 2019). Kbarr-1 yeasts were described as having the broadest antifungal spectrum due to their activity against *Hansenula, Candida,* and *Yarrowia* (Velázquez et al., 2015). Characterization of novel killer toxins is essential to withstand physical barriers to implement the killer toxins to fermentation. *Wickerhamomyces anomalus* killer toxin, called KTCf20, is active against wine spoilage yeasts (*Dekkera anomala, Brettanomyces bruxellensis, Pichia membranifaciens* and *Pichia guilliermondii*). Also, *W. anomalus* can grow in extreme environments such as high osmotic pressure and low pH (Fernández de Ullivarri et al., 2018).

Additionally, killer yeasts were described as improvers on wine quality and selected for a new strategy to enhance technological food properties (de Ullivarri et al., 2014). These strains can indirectly elevate wine aroma when they are used to avoid contaminants. For example, *Torulaspora delbrueckii* killer yeast was used to control *S. cerevisiae* contamination in wine must fermentation and increased lactone content, resulting in a wine with intense dried fruit/pastry aroma (Borren and Tian, 2021; Velázquez et al., 2015). Biocontrol ability as well as improvement of fermentation characteristics are key elements in the implementation of yeast killer systems to be selected for composing starter cultures. The distribution of killer yeasts during the fermentation process is rarely documented. In general, the proportion of killers increases during fermentation, while sensitivity decreases (Matilde Maqueda et al., 2012). Also, the magnitude of the killer effect depends on the ratio of the killer to sensitive strains, adsorbing substances, environmental conditions, sensitive strains susceptibility, inoculum size, and nitrogen availability (Matilde Maqueda et al., 2012).

Killer phenotype detection

The detection and monitoring of yeast viral killer phenotype is essential when it would be utilized in the fermentation process, as well as in the isolation of novel strains. Rosa Esteban and Nieves Rodrıguez-Cousino, (2008) developed a molecular tag based on 23S RNA replicon for wine yeasts strains to identify the viral sequence in a mixture of strains in fermentation. For killer yeast isolation, the killer activity can be evaluated in Methylene blue (MB) plates through the inhibition zone assay. The method consists of cultivating sensitive reference strains (e.g.: *S. cerevisiae* NCYC 1006; *Pichia kluyveri* CAY 15; *Candida glabrata*NCYC 388) (da Silva Portes et al., 2013) by pour plate method. The candidate strain is then inoculated onto the surface and the killer positive strain is characterized as the colony with a clear surrounding zone, or the surrounding pourplated reference culture turns blue-stained. The candidate strains can also be loaded as 4-µl aliquots of stationary-phase cultures, patched from solid cultures, or replicated onto the seeded low-pH MB plates (Matilde Maqueda et al., 2012). However, positive killer activity may be related to antimicrobial metabolites produced by the test strains. The RNA extraction and electrophoresis analysis can be used to confirm desirable killer phenotypes. The killer strains are expected to have (i) a slower-moving, ~4.6 kb band, similar in size to the dsRNA genome of L-A virus, and (ii) a faster-moving, ~2.3 kb band, sometimes similar to the genomes of M viruses (Ramírez et al., 2015). Furthermore, the dsRNA nature of the two nucleic acid molecules can be confirmed by DNAse I and RNAse A treatments, and RNAse A plus 0.5 M NaCl. The DNA disappeared after DNAse I treatment. In a second round, dsRNA bands disappeared after RNAse A treatment, while DNA remained unaffected. Finally, the RNAse A digestion in the presence of 0.5 M NaCl degrades ssRNA but not dsRNA (Cansado et al., 1999; Rodríguez-Cousiño et al., 2011a). After electrophoresis analysis and enzyme treatments to confirm genomic characteristics, dsRNA bands can be purified and sequenced by NGS techniques (Figure 1) (Crucitti et al., 2022). More recently, Quintero-Blanco & Juan Jimenez, (2022) developed an accurate method to rapid diagnosis of killer dsRNA totiviruses and variants of *S. cerevisiae* strains based on an RT-PCR multiplex assay. Research on adaptations of methodologies can simplify the processes for greater efficiency in industrial viral identification.

Patent landscape

The comprehension of the yeast 'killer factor' as a coevolutional mechanism with implications in microbial ecological interactions unveiled a new perspective for the food industry: selection of starter cultures capable of producing exotoxins for the biopreservation of fermented foods and beverages (Ciani and Comitini, 2015; Kordialik-Bogacka, 2022; Mannazzu et al., 2019). Over the past decade, killer yeasts became the subject of numerous research, building up enough basic knowledge to lead the transference of technology from the scientific sphere to commercial applications (Table 2).

Table 2. Viral killer system patents.

Document number	Strain/Toxin genes	Interest
US20210284939A1	K1, K2, K28	Beer production
US10306898B2	*S. bayanus* and non-*Saccharomyces* strains	Shelf-life of bread, dough, and baked goods
US20220039402A1	*S. bayanus* and non-*Saccharomyces* strains	Shelf-life of bread, dough, and baked goods
US20200048592A1	toxin-producing *S. cerevisiae* and *S. bayanus*	Wine making

Font: Patent Inspiration and Espacenet Patent Search.

A survey on free-access patent databases (Patent Inspiration and Espacenet Patent Search) revealed four documents filled over the past five years protecting the development and/or use of killer yeasts in the food industry. The US20210284939A1 document refers to the control of the attenuation in beer production through the addition of selected or modified yeasts bearing the K1, K2, or K28 toxin genes. Attenuation is the undesirable fermentation of long-chain oligosaccharides (e.g.: dextrin; starch) by the spoilage *S. cerevisiae var. diastaticus*, resulting in gas accumulation, reduction of specific gravity, and production of off-flavors in the final product (Štulíková et al., 2021). According to the applicant Rhinegeist LLC., the addition of a 'killer' *S. cerevisiae* was able to eliminate the diastatic yeast based on real-time monitoring of the specific gravity. Recent patents (US10306898B2; US20220039402A1) also disclosed the selection of the 'killer' trait in *S. bayanus* and non-*Saccharomyces* for improving the shelf-life of bread, dough, and baked goods. The production and/or resistance to exotoxins has become a

mandatory characteristic for mixed fermentation processes, such as wine, to ensure the dominance of the strain over wild yeasts and the survival of other toxin-producing starter cultures (Comitini et al., 2021). Italian enterprise Bioenologia 2.0 S.r.l. protected the *S. bayanus subsp. uvarum* strain SIRIUS based on the absence of mutual exclusion concerning 10 oenological, toxin-producing *S. cerevisiae* and *S. bayanus* (US20200048592A1). This selection model has also been adopted by other companies from the wine market, such as Oenobrands® and Brouwland, who already provide *S. cerevisiae var. cerevisiae* and *S. cerevisiae var. bayanus* with the 'killer' trait.

Conclusion remarks and future perspectives

Most eukaryotic viruses in fermented foods are dsRNA of the family *Totiviridae* that mainly infect *Saccharomyces* spp. Wine is the most frequently studied fermented food for this topic, evidencing the lack of studies for other fermented foods. These viruses do not appear to cause any harm to human cells or the gastrointestinal microbiota. Instead, they have prominent applications in food technology and potential of being implemented as starter cultures. The killer toxins secretion can prevent unwanted microbial spoilage species of fungi and bacteria. Exploring viral diversity and physiology among yeasts viruses can provide a more effective and broader role as biocontrol agents to the food industry. Additionally, when a killer system is present, it can result in improvement of sensory quality of fermentation by increasing the biosynthesis of aromatic compounds and related compounds. Viruses can impact fermented food and beverage by utilizing their host cellular machinery. This relationship is a profound endosymbiosis where the virus utilizes yeast machinery cells to propagate and, on the other side, secretion of killer toxins is an advantage on yeast's intraspecific competition. Genomic and metagenomic data proves co-evolution between viruses and cells. Advances in sequencing technology and comparative genomics have expanded our understanding of the evolutionary relationships between viruses and cellular organisms. Despite recent advances in NGS, viromes remain poorly described in the scientific database. Improvement of NGS technology to embrace viruses in food diversity studies is of utmost importance. These tools are essential to expanding food technology and food safety all over eukaryotic mycoviruses.

CHAPTER SIX – COMPREHENSIVE METAGENOMIC ANALYSIS OF VINEGAR FERMENTATION REVEALS SIGNIFICANT BACTERIA-VIRUS INTERACTIONS AND FUNCTIONAL INSIGHTS

Manuscript under review for publication in the journal Beverages (MDPI).

Abstract

This study employed shotgun metagenomics to investigate microbial dynamics, phage-bacteria interactions, and functional genes throughout a three-month apple vinegar fermentation process. A total of 5,621 microbial species were identified, revealing three distinct phases: (i) Enterobacteria and non-*Saccharomyces* species dominating the initial substrate; (ii) *S. cerevisiae* and *Leuconostoc pseudomesenteroides* prevailing in the intermediate phase; and (iii) acetic acid bacteria (*Acetobacter ghanesis* and *Gluconobacter* spp.), alongside non-*Saccharomyces* species (*Pichia kudriavzevii* and *Malassezia restricta*), dominating the final stages. Bacteriophage analysis revealed the presence of phages targeting spoilage bacteria, such as *Pseudomonas* and *Erwinia*, suggesting a role in regulating microbial stability and enhancing fermentation control. Functional metagenomic analysis highlighted key pathways associated with microbial growth and metabolite production, including carbohydrate and amino acid metabolism, energy production, and glycan biosynthesis. Enzymes involved in stress adaptation and secondary metabolism, including oxidative phosphorylation and phenolic compound synthesis, revealed microbial resilience and their potential role in shaping the product's sensory and functional properties. Moreover, *Enterobacteriaceae* species were associated with pectin degradation during the early stages, aiding substrate breakdown. These findings are crucial for microbial and phage management in fermentation technology, offering valuable insights for innovation in the vinegar industry.

Keywords: Vinegar quality; Microbiome; Phage therapy in fermentation; Shotgun sequencing; KEGG analysis.

Introduction

Vinegar fermentation is a complex bioprocess driven by a dynamic core microbiome, primarily composed of yeasts, acetic acid bacteria (AAB), and lactic acid bacteria (LAB) (Mas et al., 2015). However, the presence of less-characterized microorganisms and their enzymatic functions also play a crucial role (Mota and Vilela, 2024). These microbial transitions not only influence the final acidity and flavor profile but also affect the nutritional content and health benefits of the vinegar (Maske et al., 2024a). Furthermore, the microbial diversity during fermentation is closely linked to the accumulation of secondary metabolites and aroma compounds, which enhance the overall quality of the vinegar (Ferrocino et al., 2018). Understanding how microbial succession impacts these processes can lead to the development of targeted strategies to optimize fermentation conditions and create vinegar with specific flavor profiles and health-promoting properties (Marco and Abram, 2019).

Advances in metagenomics have significantly enhanced our understanding of the microbial diversity that drives fermentation processes (Zhang et al., 2021). Shotgun metagenomics, a high-throughput sequencing approach, identifies microbial species, analyzes functional genes, and explores metabolic networks in a single step, providing a detailed view of the microbial ecosystem (Román-Camacho et al., 2024; Tamang et al., 2021). This approach has facilitated the development of strategies to optimize the simulation of microbial consortia and achieve fermented products with enhanced quality, stability, and functional properties (Weiland-Bräuer, 2021).

In vinegar research, shotgun metagenomics was initially employed to investigate the microbiota of cereal vinegar, revealing the metabolic networks responsible for its distinct flavor (Román-Camacho et al., 2024; Tamang et al., 2021). This analysis highlighted the microbial diversity and functional contributions of key species, especially

yeast and AAB, in shaping the sensory characteristics of the product. Additionally, it facilitated the reconstruction of metabolic pathways and provided insights into microbial interactions and the adaptation mechanisms of the vinegar microbiome under specific production conditions (Román-Camacho et al., 2024). Despite these advancements, critical knowledge gaps remain, particularly regarding the complex interactions between bacteria, viruses, and other microorganisms involved in vinegar fermentation. This is particularly evident in vinegars produced from diverse substrates and origins, where differences in raw materials and environmental conditions further complicate the understanding of microbial dynamics and their impact on fermentation processes

Understanding bacteria-virus interactions in fermentation is critical due to their dual role in microbial ecosystems (Weiland-Bräuer, 2021). Controversial hypotheses suggest that bacteriophages could serve as natural biocontrol agents, controlling spoilage microbes and enhancing microbial stability (Ranveer et al., 2024). However, their presence has also been linked to reduced fermentation efficiency by infecting key bacterial species. Additionally, the functional roles of underexplored microorganisms and their enzymatic contributions to traditional apple vinegar fermentation, such as pectin degradation and aromatic compound production, have yet to be fully understood. This can be addressed through Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis, which offers comprehensive insights into metabolic pathways, enzymatic functions, and microbial interactions (Ogata et al., 1998).

This study aimed to provide a comprehensive metagenomic analysis of apple vinegar fermentation, focusing on microbial diversity, bacteria-phage interactions, and functional pathways. By exploring these elements, the work uncovers critical insights into the fermentation process, offering new perspectives on microbial ecosystem management and paving the way for optimizing fermentation conditions. These findings contribute to

enhancing both product stability and sensory quality, advancing the broader understanding of this ancient fermentation practice.

Material and Methods

Vinegar production and sampling

Apple vinegar fermentation was carried out following the protocol described by Maske et al. (2024b), with all assays performed in triplicate according to artisanal methods traditionally practiced in Brazilian domestic settings. Fresh organic apples were sourced from a local market in Curitiba, Paraná, Brazil, and selected based on uniformity, ripeness, and the absence of visible defects. The apples were processed at a ratio of 0.5 kg per liter of must. The must was manually prepared by crushing the apples and mixing them with sterile water. Refined white sugar was then added to adjust the initial soluble solids concentration to 20 ◦Brix, as measured using a digital refractometer (Hanna Instruments, Woonsocket, RI, USA) thereby ensuring suitable conditions for spontaneous alcoholic fermentation.

Fermentations were conducted in sterile 3 glass containers and incubated for 90 days, comprising two distinct phases. The first phase consisted of spontaneous alcoholic fermentation, which lasted six weeks. Following this period, an acetic inoculum (known as “mother vinegar”) was introduced to initiate the second phase—induced acetic fermentation (Maske et al., 2024b). Samples of 50 mL were collected on days 0, 30, 60, and 90, immediately frozen to preserve their integrity, and stored at −20 ◦C until microbiological analysis.

Total DNA extraction

Genomic DNA from the microbial community was extracted from 10 mL of each sample collected during fermentation. The samples were centrifuged at 4,000× g, 4 °C, for 10 minutes to pellet the microbial cells, which were then resuspended in 200 µl of phosphate-buffered saline (PBS). To facilitate cell wall disruption, the resuspended material was treated with specific enzymes: lysozyme (10 mg/mL) to break down bacterial cell walls and lyticase (2 mg/mL) to degrade the yeast cell walls. Following enzyme treatment, the DNA was purified using the DNeasy PowerSoil kit (Qiagen), according to the manufacturer's instructions. The extracted DNA was quantified using a Nanodrop spectrophotometer (Thermo Fisher Scientific).

The extracted DNA was quantified using a Nanodrop spectrophotometer (Thermo Fisher Scientific) and assessed for integrity via 0.8% (w/v) agarose gel electrophoresis.

Viral DNA extraction

To recover the viral particles, 10 ml of apple cider vinegar was centrifuged at 500 g for 5 minutes to remove the particulate fraction from the sample. The supernatant was then filtered using a 0.22 µm syringe filter. RNase A and DNase I were added to the filtrate at a final concentration of 1 µg/mL and left at 37 °C for 30 min to degrade the genomic DNA and RNA of the microbial cells. To precipitate the viral particles, a cold solution of PEG 8000 was added to the sample to reach a final concentration of 10% (w/v) and incubated at 4 °C overnight. The mixture was then centrifuged at 12,000× g, 4 °C, for 1 hour and the supernatant was discarded. The precipitated material was then resuspended in 2 mL of ice-cold SM buffer (200 mM NaCl, 10 mM MgSO4, 50 mM Tris pH 7.5) and stored at 4 °C until use.

The nucleic acids of the purified viruses were extracted using the Viral DNA/RNA Extraction kit (Loccus). After treating part of the extract with DNAse, the RNA was

converted into cDNA using the High-Capacity RNA-cDNA kit (ThermoFisher) and quantified using Nanodrop spectrophotometer (Thermo Fisher Scientific).

Library construction and shotgun sequencing

For microbial DNA, 1 ng of genomic DNA was used to construct the library using the Nextera XT DNA Library Preparation Kit (Illumina), following the manufacturer's protocol. For viral DNA, both the extracted DNA and complementary DNA (cDNA) were combined in equimolar proportions to construct the viral library, using the same preparation procedure as described for microbial DNA. The quality and size distribution of the libraries were assessed using GelBot (Loccus). Sequencing was performed on the Illumina NextSeq platform, employing a paired-end protocol with a read length of 300 base pairs (2 × 150 bp).

Bioinformatics and data analysis

For each sample, quality control of the sequences generated by sequencing was carried out using the Trimmomatic v.0.39 software (Bolger et al., 2014). This procedure aims to remove low-quality regions and adapter sequences that could affect the quality of the genome assembly. After this stage, the reads from each sample were used to assemble the metagenome using the SPAdes v.3.15.4 algorithm with the -meta parameter (Bankevich et al., 2012). SPAdes v.3.15.4 is a robust and widely used algorithm for assembling metagenomes, as it can deal with sequences of different sizes and complexities. To ensure the accuracy and quality of the metagenome annotation, the Prokka v.1.14.6 tool (Seemann, 2014) was used. This tool is capable of automatically annotating genes and other characteristics, providing information on the genetic

composition of the sample. In addition, the Kraken taxonomic sequence classifier, which is based on complete bacterial, fungal, archaeal, plant, and viral genomes in the National Center for Biotechnology Information (NCBI) RefSeq database, was used to classify the sequences (Wood and Salzberg, 2014). However, only sequences related to fungi, bacteria, and bacteriophages were considered, as they are key drivers of microbial interactions and functional dynamics in vinegar fermentation.

eggNOG, KEGG, and pathway annotation

The translated gene sequences were aligned against the eggNOG protein database using BLASTP (WU-BLAST 2.0; http://blast.wustl.edu). A single gene could be assigned to multiple functional categories within eggNOG, as described by Xie et al., (2013). Annotation based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) was performed using the KAAS tool with default parameters in Single Best Hit mode (http://www.genome.jp/kegg/kaas/). Genes associated with KEGG orthology were subsequently submitted to iPath (http://pathways.embl.de) for metabolic pathway reconstruction in each sample.

Results and Discussion

Dynamic succession of bacteria and fungi (Shotgun reads)

A total of 1,860,561 reads were obtained from traditional apple vinegar samples. Shotgun metagenomic sequencing identified a diverse microbial community, encompassing 44 phyla, 221 orders, and 5,621 species across bacterial and fungal domains. The proportion of unclassified taxa significantly increased as taxonomic classification advanced to deeper levels (Figure 1). For bacteria, at the phylum level, only

4.1 % of taxa remained unclassified by Day 90. However, at the order and species levels, the proportion of unclassified taxa reached 13.2% and 72.7%, respectively. Similarly, for fungi, unclassified taxa at the phylum level accounted for 37.3% by Day 90, increasing to 39.58% at the order level and 53.5% at the species level. The observation of increasing proportions of unclassified taxa at finer taxonomic levels in metagenomic studies is a well-documented phenomenon. For instance, a study evaluating metagenomic classifiers on soil microbiomes found a substantial proportion of unclassified reads (Edwin et al., 2024). Similarly, a taxonomic profiling of ancient metagenomic samples reported that a significant proportion of reads remained unclassified across various metagenomic profilers (Pusadkar and Azad, 2023). These findings align with our observations, where unclassified taxa percentages increased from broader to finer taxonomic levels. The high proportion of unclassified taxa emphasizes the need for expanded genomic databases and advanced sequencing technologies to improve taxonomic resolution. Despite this limitation, the functional potential of unclassified taxa remains significant, as they may harbor unique metabolic pathways crucial for food fermentation processes. This highlights the importance of integrating functional analyses with taxonomic studies to better understand and optimize microbial roles in enhancing food flavor, texture, and preservation during fermentation.

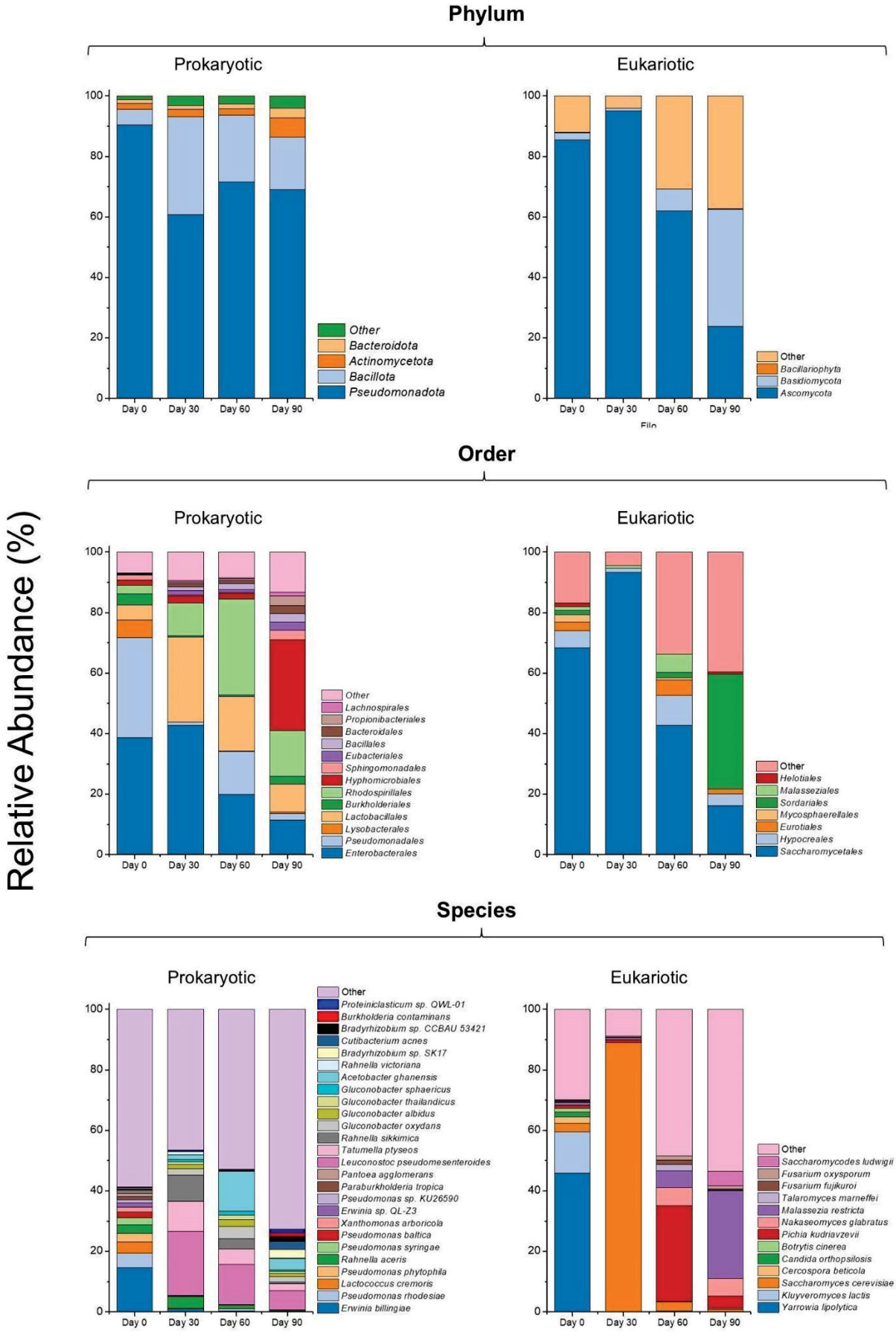

Phylum
Prokaryotic
Eukariotic
Relative Abundance (%)
Day 0
Day 30
Day 60
Day 90
Other
Bacteroidota
Actinomycetota
Bacillota
Pseudomonadota
Other
Bacillariophyta
Basidiomycota
Ascomycota
Order
Other
Lachnospirales
Propionibacteriales
Bacteroidales
Bacillales
Eubacteriales
Sphingomonadales
Hyphomicrobiales
Rhodospirillales
Burkholderiales
Lactobacillales
Lysobacterales
Pseudomonadales
Enterobacterales
Other
Helotiales
Malasseziales
Sordariales
Mycosphaerellales
Eurotiales
Hypocreales
Saccharomycetales
Species
Other
Proteiniclasticum sp. QWL-01
Burkholderia contaminans
Bradyrhizobium sp. CCBAU 53421
Cutibacterium acnes
Bradyrhizobium sp. SK17
Rahnella victoriana
Acetobacter ghanensis
Gluconobacter sphaericus
Gluconobacter thailandicus
Gluconobacter albidus
Gluconobacter oxydans
Rahnella sikkimica
Tatumella ptyseos
Leuconostoc pseudomesenteroides
Pantoea agglomerans
Paraburkholderia tropica
Pseudomonas sp. KU26590
Erwinia sp. QL-Z3
Xanthomonas arboricola
Pseudomonas baltica
Pseudomonas syringae
Rahnella aceris
Pseudomonas phytophila
Lactococcus cremoris
Pseudomonas rhodesiae
Erwinia billingiae
Other
Saccharomycodes ludwigii
Fusarium oxysporum
Fusarium fujikuroi
Talaromyces marneffei
Malassezia restricta
Nakaseomyces glabratus
Pichia kudriavzevii
Botrytis cinerea
Candida orthopsilosis
Cercospora beticola
Saccharomyces cerevisiae
Kluyveromyces lactis
Yarrowia lipolytica

Figure 1. Relative abundance of microbial taxa at the phylum, order, and species levels during apple vinegar fermentation over 90 days. Prokaryotic and eukaryotic communities are shown separately, with distinct taxonomic distributions at each level.

Microbial diversity

Across all fermentation stages (Day 0, Day 30, Day 60, Day 90), the bacterial and fungal communities were dominated by distinct phyla (Figure 1). In bacteria, Proteobacteria and Firmicutes were the most abundant, with Proteobacteria being dominant initially (58.62%) and gradually decreasing by Day 90 (46.41%), while Firmicutes increased significantly over time (14.65% at Day 0 to 72.73% at day 90). For fungi, Ascomycota was consistently dominant, particularly at Day 30 (89.02%), accounting for most fungal taxa across all time points. These shifts influence metabolic pathways and sensory outcomes in vinegar production, with the roles of key microorganisms explored further in the following topics.

Order-level trends

At the bacterial order level, the initial stages of fermentation (Day 0) were dominated by *Enterobacterales* (38.6%) and *Pseudomonadales* (33.1%), representing taxa typically associated with nutrient-rich environments and rapid growth (LaBauve and Wargo, 2012). However, this composition changed markedly by Day 30, with *Lactobacillales* emerging as the dominant phylum (27.9%), likely due to its ability to thrive in acidic conditions created during fermentation (Osborne, 2010). By Day 60, a new shift occurred with *Rhodospirillales* becoming dominant (31.5%), reflecting the adaptive capacity of taxa such as *Acetobacteraceae*, a family well-suited for survival and activity in highly acidic and oxygenated environments (Niyomvong et al., 2022; Qiu et al., 2021). By Day 90, the microbial community displayed significant diversity, with no single phylum dominating. Diverse orders, including *Hyphomicrobiales, Lactobacillales*,

and *Rhodospirillales*, were present. This diversification likely resulted from the depletion of easily fermentable substrates and the accumulation of secondary metabolites (Maske et al., 2024b), which may have inhibited dominant taxa and facilitated the coexistence of less competitive yet specialized microbes.

For fungi, the initial stage (Day 0) was dominated by *Saccharomycetales* (68.4%), which includes many yeast species adapted to high-sugar environments (Erasmus et al., 2003). However, by Day 30, there was a near-complete dominance of *Saccharomyces cerevisiae* within this phylum, comprising 89.02% of the total fungal population. This is consistent with the rapid proliferation of fermentative yeasts during the early stages of fermentation when sugars are abundant (Dashko et al., 2014). As fermentation progressed, the fungal community also exhibited high diversity, with major contributions from *Hypocreales* (Day 60), Eurotiales (Day 90), and other minor phyla, suggesting a stabilization phase where specialized fungi occupy diverse ecological niches.

Species-level interactions

The succession of microbial species during natural fermentation for vinegar production revealed dynamic shifts in bacterial and fungal communities (Figure 1), strongly influenced by the evolving fermentation environment. At bacterial level, the early dominance of *Erwinia billingiae* (14.65%) and *Pseudomonas rhodesiae* (4.76%) reflects the initial presence of environmental and opportunistic taxa. These species are often associated with fresh substrates and aerobic conditions but tend to diminish as fermentation progresses (Karanth et al., 2023).

The first significant microbial shift was detected after 30 days, marked by the consistent dominance of fermentative microorganisms such as *Saccharomyces cerevisiae* (89%), *Leuconostoc pseudomesenteroides* (21.2%). The intricate nature of LAB-yeast

interactions is underscored by two key mechanisms: (i) yeast autolysis releases essential nutrients, including amino acids, polysaccharides, and riboflavin, which support bacterial growth, and (ii) the acidification of the fermentation medium by LAB creates a favorable environment for yeast proliferation (de Oliveira Junqueira et al., 2019). These synergistic interactions have been shown to enhance sensory characteristics in products like wine, sourdough, and yogurt (Fleet, 2003). However, detailed insights into these mechanisms during vinegar fermentation remain limited.

The rise of *Leuc. pseudomesenteroides* during Days 30 and 60, peaking at 21.5% and 13.11%, respectively, highlights its critical role in the early and intermediate stages of fermentation. This species is known for its lactic acid production, which contributes to pH reduction, creating a more selective environment for acid-tolerant species (Maske et al., 2024b). The dominance of *S. cerevisiae* on Day 30 highlights its key role in ethanol fermentation and competitiveness in sugar-rich environments. The high production of ethanol and lactic acid during this phase creates strong selective pressures favoring organisms capable of maintaining activity under extreme environmental conditions (Atasoy et al., 2024).

By Day 60, the emergence of acid-tolerant, AAB, became essential for the oxidation of ethanol to acetic acid, a critical step in vinegar production (Nie et al., 2013). The dominant species was *Acetobacter ghanensis*, accounting for 13.07% of the reads; however, a broad diversity of AAB was reported, including *Gluconobacter oxydans, G. albidus*, *G. thailandicus*, and *G. sphaericus*. This high diversity can result in the accumulation of various metabolites in addition to acetic acid, playing an essential role in the development of natural vinegar aromas. Studies on individual AAB species can help develop microbial blends to produce vinegars with better sensory profiles, richer aromas, and tailored functional properties for various consumer and industrial needs

Interestingly, *Pichia kudriavzevii* emerged with a transient dominance (31.5%) following the alcoholic phase dominated by *S. cerevisiae.* The reduction in ethanol, driven by AAB activity, may have allowed this yeast to thrive. Known for its resilience, *P. kudriavzevii* can tolerate high levels of acetic acid, low pH, and other fermentation stressors (N. Wang et al., 2023), making it a competitive organism in this phase. Its presence has been linked to the production of desirable aromatic compounds that enhance the sensory profile of fermented products (Pereira et al., 2017). Further investigations could focus on selecting this species as an adjunct in vinegar fermentation to enhance aromatic complexity and improve sensory attributes.

Malassezia restricta emerged strongly at 90 days, coinciding with reduced ethanol levels and the availability of complex substrates like lipids (Maske et al., 2024b). Traditionally associated with the skin microbiota (Vijaya Chandra et al., 2021), *M. restricta* is a lipophilic yeast capable of utilizing lipid-rich substrates, likely derived from cell debris or fermentation by-products. Its growth indicates that the fermentation environment shifted to favor microbes adapted to such conditions. These microbial shifts have direct implications for the quality of the final vinegar, as they may influence the development of unique aromatic compounds, contribute to the complexity of the sensory profile, and affect the overall stability and functionality of the product.

Bacteriophage diversity

The virome analysis revealed over 25 bacteriophage sequences classified within the order Caudovirales (Figure 2), with the viral families *Myoviridae*, *Podoviridae*, and *Siphoviridae* being the most abundant. The *Myoviridae* family, recognizable by their long, contractile tails, included notable bacteriophages such as *Pseudomonas phage PMBT3*, *Pseudomonas phage UJF_PDIM6*, and *Erwinia phage vB_Ems49*. These

phages, capable of infecting a diverse range of bacterial hosts, play a pivotal role in shaping microbial communities vital for vinegar production (Naureen et al., 2020). On the other hand, the *Podoviridae* family, defined by their short, non-contractile tails, included phages such as *Pseudomonas* phage phiAH14a, *Lactococcus* phage Tuc2009, and *Gordonia* phage Puppar, which are known for their efficiency in rapidly lysing bacteria and significantly influencing microbial succession dynamics during the fermentation process (Warriner and Namvar, 2019). In contrast, the *Siphoviridae* family, distinguished by their long and flexible tails, comprised phages such as *Erwinia* phage vB_Ems58, *Mycobacterium* phage Noxifer, and *Mycobacterium* phage PurpleHaze. These phages often establish stable, host-specific interactions, which help sustain microbial diversity within the fermentation environment. Additionally, several bacteriophages could not be assigned to these families and were categorized as 'unclassified *Caudoviricetes*.' This group included less abundant phages from the genera *Pagevirus* and *Lessievirus*, along with others exhibiting unique or unidentified characteristics.

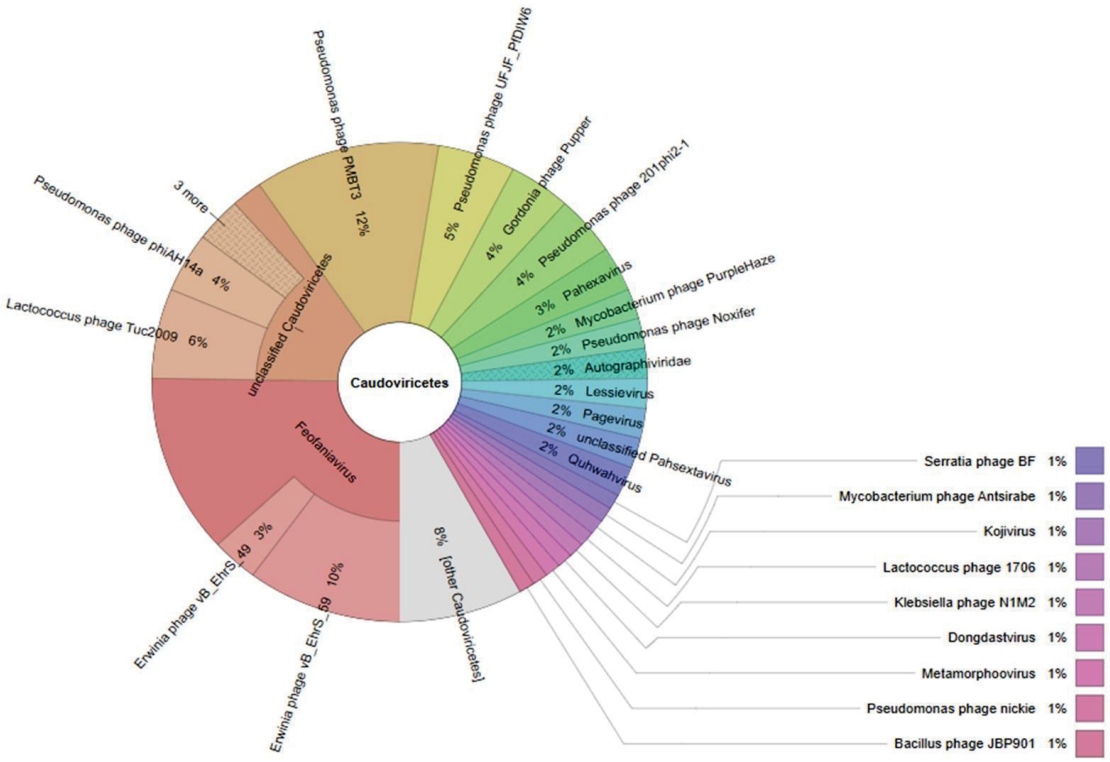


Figure 2. Phage viruses detected in vinegar after three months of natural fermentation.

Phage-bacteria interactions

Phages displayed a wide spectrum of host specificities, with the majority targeting a single bacterial host. The predominant phages identified were *Pseudomonas phage PMBT3* (12% of total phage reads), *Erwinia phage vB_Ems49* (10%), *Erwinia phage vB_Ems58* (8%), and *Lactococcus phage Tuc2009* (6%) (Figure 2). To the best of our knowledge, this is the first study to identify *Pseudomonas phage PMBT3* within the virome diversity of vinegar samples. This phage has garnered attention for its potential as a biocontrol agent against *P. aeruginosa* and its biofilms, which are of particular concern in the food industry (Hylling et al., 2020; Yang et al., 2025). Moreover, its application could be further explored in vinegar production, where the control of microbial contaminants is critical to ensuring product quality and safety. By targeting specific bacterial pathogens, PMBT3 may offer a sustainable and efficient approach to

enhance the overall microbiological stability of vinegar fermentation processes. A diverse array of other lytic bacteriophages targeting *Pseudomonas* species was identified in this study. Among these, the lytic bacteriophage *Pseudomonas* UFJF_PfDIW6 (5%) has gained significant attention for its reported antimicrobial activity against various *Pseudomonas* strains under different pH levels and temperatures (Hungaro et al., 2022; Nascimento et al., 2022). Additionally, the presence of phages such as 201ϕ2-1 (targeting both *Pseudomonas chlororaphis* and *P. aeruginosa*), Noxifer (*P. aeruginosa*), and Nickie (*Pseudomonas syringae pv. avii*) underscores the genus' phage diversity and its potential for biocontrol and quality improvement (Kwon et al., 2021; Martino et al., 2021; Morozova et al., 2023; Thomas et al., 2008).

The second most prevalent group of phages targeted *the Erwinia* species, representing 13% of the total reads. Among these, two temperate phages, *Erwinia phage Vb_EhrS_59* (10%) and *Erwinia phage Vb_EhrS_49* (3%), were identified (Figure 2). These phages share substantial nucleotide sequence identity, particularly in genes associated with head assembly, DNA packaging, and lysis (Zlatohurska et al., 2019). Notably, both phages infect *Erwinia horticola*, the pathogen responsible for beech black bacteriosis, highlighting their potential as biocontrol agents. Moreover, their lack of significant similarity to previously characterized viruses within the *Enterobacteriaceae* family underscores their uniqueness and opens avenues for novel biotechnological applications.

Interestingly, no viruses infecting yeast and key AAB were detected in this study. However, the analysis identified phages targeting LAB, such as *Lactococcus phage Tuc2009* (6%) and another *Lactococcus* phage (1%), which could threaten LAB persistence. Although *Lactococcus* does not play a central role in the final stages of vinegar production, it may contribute to the vinegar's flavor profile. The presence of LAB

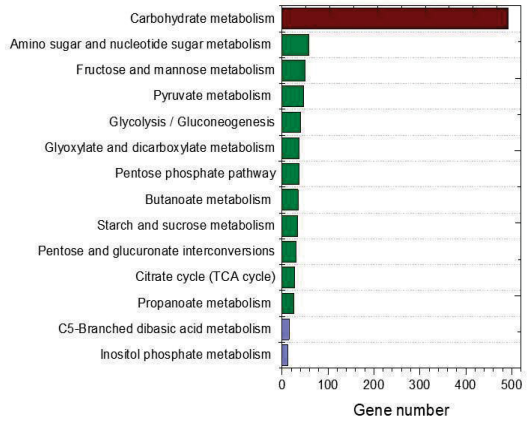
phages in fermentation systems poses risks by disrupting bacterial growth and delaying fermentation, potentially leading to lower-quality products (Ranveer et al., 2024).

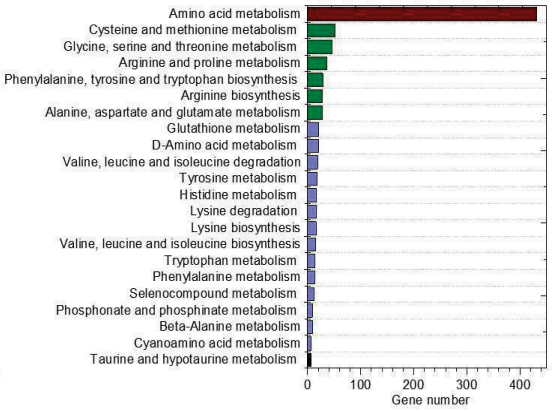
The investigation of the minor viral population revealed phages targeting pathogenic bacteria in apple cider vinegar, including *Pahexavirus* (3%), which infects *Enterobacteriaceae*, as well as *Serratia phage BF* (1%) and *Klebsiella phage N1M2* (1%). *Pahexavirus* has demonstrated anti-biofilm activity against *Cutibacterium acnes*a—bacterium known to contaminate traditional fermented foods through human handling (Li et al., 2024; Mayslich et al., 2021). This finding underscores the broader impact of phage populations in fermentation environments, not only in shaping microbial communities but also in offering potential applications for targeted biocontrol strategies.

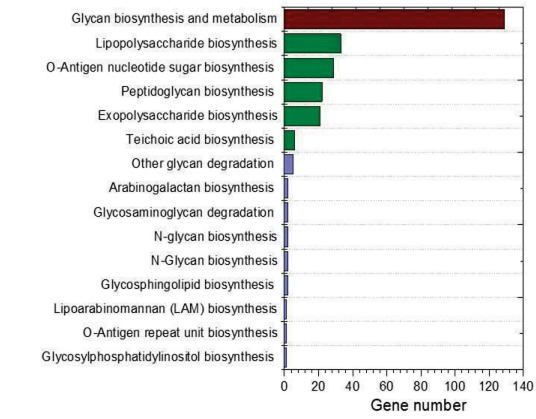
Predictive functional features

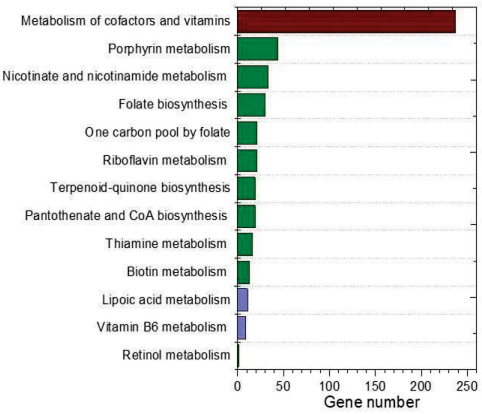
The functional annotation of the metagenomic sequences revealed that 57% of the genes were assigned to Clusters of Orthologous Groups (COG; a database categorizing gene based on orthology and conserved functions across species), while the remaining 43% of open reading frames were mapped to the Kyoto Encyclopedia of Genes and Genomes (KEGG; a database linking genes to metabolic pathways and higher-level biological systems). The most abundant COG category, “general function prediction only”, highlighted genes with versatile and broadly defined roles (Sinha et al., 2020). Amino acid and carbohydrate transport were also significant, underscoring their role in microbial growth, flavor compound production, and energy utilization during fermentation (Wei et al., 2023). Additional COG categories, such as transcription, ribosomal biogenesis, energy production, and cell envelope biogenesis, emphasized the community's capacity to adapt and maintain structural resilience in the harsh fermentation environment (Jeckelmann and Erni, 2020).

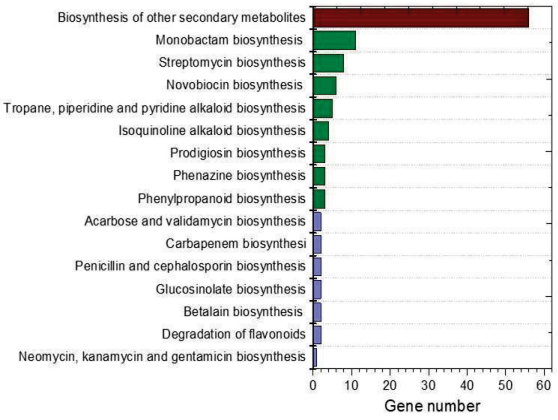
The KEGG metabolic pathway analysis, as reported in Figure 3, categorized genes into three hierarchical levels: Level 1, representing broad high-level functions such as metabolism, environmental information processing, and genetic information processing (100%); Level 2, encompassing specific functional categories such as carbohydrate metabolism, amino acid metabolism, and energy metabolism (5-30%); and Level 3, detailing individual sub-pathways contributing to metabolic and functional diversity (1-10%).

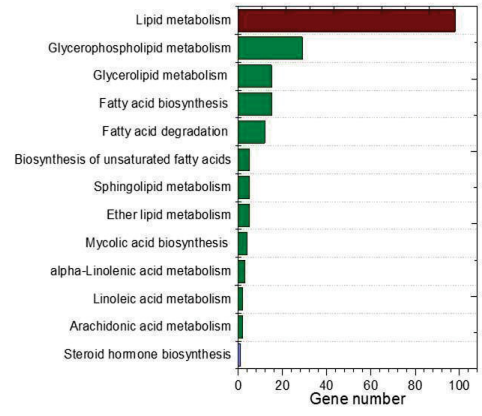

Carbohydrate metabolism
Amino sugar and nucleotide sugar metabolism
Fructose and mannose metabolism
Pyruvate metabolism
Glycolysis / Gluconeogenesis
Glyoxylate and dicarboxylate metabolism
Pentose phosphate pathway
Butanoate metabolism
Starch and sucrose metabolism
Pentose and glucuronate interconversions
Citrate cycle (TCA cycle)
Propanoate metabolism
C5-Branched dibasic acid metabolism
Inositol phosphate metabolism
0 100 200 300 400 500
Gene number

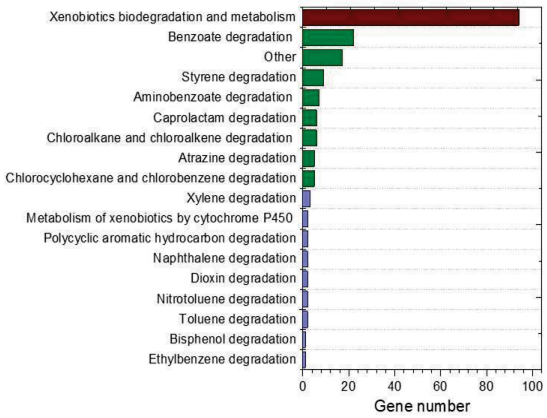

Amino acid metabolism
Cysteine and methionine metabolism
Glycine, serine and threonine metabolism
Arginine and proline metabolism
Phenylalanine, tyrosine and tryptophan biosynthesis
Arginine biosynthesis
Alanine, aspartate and glutamate metabolism
Glutathione metabolism
D-Amino acid metabolism
Valine, leucine and isoleucine degradation
Tyrosine metabolism
Histidine metabolism
Lysine degradation
Lysine biosynthesis
Valine, leucine and isoleucine biosynthesis
Tryptophan metabolism
Phenylalanine metabolism
Selenocompound metabolism
Phosphonate and phosphinate metabolism
Beta-Alanine metabolism
Cyanoamino acid metabolism
Taurine and hypotaurine metabolism
0 100 200 300 400
Gene number


Glycan biosynthesis and metabolism
Lipopolysaccharide biosynthesis
O-Antigen nucleotide sugar biosynthesis
Peptidoglycan biosynthesis
Exopolysaccharide biosynthesis
Teichoic acid biosynthesis
Other glycan degradation
Arabinogalactan biosynthesis
Glycosaminoglycan degradation
N-glycan biosynthesis
N-Glycan biosynthesis
Glycosphingolipid biosynthesis
Lipoarabinomannan (LAM) biosynthesis
O-Antigen repeat unit biosynthesis
Glycosylphosphatidylinositol biosynthesis
0 20 40 60 80 100 120 140
Gene number


Metabolism of cofactors and vitamins
Porphyrin metabolism
Nicotinate and nicotinamide metabolism
Folate biosynthesis
One carbon pool by folate
Riboflavin metabolism
Terpenoid-quinone biosynthesis
Pantothenate and CoA biosynthesis
Thiamine metabolism
Biotin metabolism
Lipoic acid metabolism
Vitamin B6 metabolism
Retinol metabolism
0 50 100 150 200 250
Gene number


Biosynthesis of other secondary metabolites
Monobactam biosynthesis
Streptomycin biosynthesis
Novobiocin biosynthesis
Tropane, piperidine and pyridine alkaloid biosynthesis
Isoquinoline alkaloid biosynthesis
Prodigiosin biosynthesis
Phenazine biosynthesis
Phenylpropanoid biosynthesis
Acarbose and validamycin biosynthesis
Carbapenem biosynthesi
Penicillin and cephalosporin biosynthesis
Glucosinolate biosynthesis
Betalain biosynthesis
Degradation of flavonoids
Neomycin, kanamycin and gentamicin biosynthesis
0 10 20 30 40 50 60
Gene number


Lipid metabolism
Glycerophospholipid metabolism
Glycerolipid metabolism
Fatty acid biosynthesis
Fatty acid degradation
Biosynthesis of unsaturated fatty acids
Sphingolipid metabolism
Ether lipid metabolism
Mycolic acid biosynthesis
alpha-Linolenic acid metabolism
Linoleic acid metabolism
Arachidonic acid metabolism
Steroid hormone biosynthesis
0 20 40 60 80 100
Gene number


Xenobiotics biodegradation and metabolism
Benzoate degradation
Other
Styrene degradation
Aminobenzoate degradation
Caprolactam degradation
Chloroalkane and chloroalkene degradation
Atrazine degradation
Chlorocyclohexane and chlorobenzene degradation
Xylene degradation
Metabolism of xenobiotics by cytochrome P450
Polycyclic aromatic hydrocarbon degradation
Naphthalene degradation
Dioxin degradation
Nitrotoluene degradation
Toluene degradation
Bisphenol degradation
Ethylbenzene degradation
0 20 40 60 80 100
Gene number

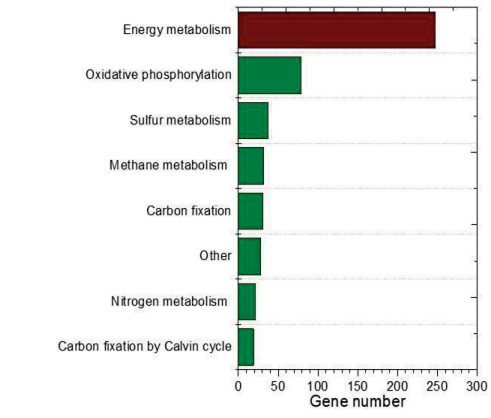

Energy metabolism
Oxidative phosphorylation
Sulfur metabolism
Methane metabolism
Carbon fixation
Other
Nitrogen metabolism
Carbon fixation by Calvin cycle
0 50 100 150 200 250 300
Gene number

Figure 3. Functional classification of genes based on KEGG pathways during natural vinegar fermentation. The distribution of genes is highlighted in red (Level 1; 100%), green (Level 2; 5–30%), and blue (Level 3; 1–10%).

At Level 1, metabolism emerged as the most dominant high-level function, followed by environmental information processing, genetic information processing, and cellular processes (Figure 3). A smaller fraction of sequences was classified as poorly characterized, reflecting limited functional information for certain genes. Within the high-level metabolism category, carbohydrate metabolism represented the most abundant function, accounting for 13.54% of annotated genes, followed by amino acid metabolism with 11.89% (Figure 3). Similarly, a study by Liu et al., (2020) found that genes related to carbohydrate and amino acid metabolism were highly expressed in soybean paste fermentation, emphasizing their roles in energy generation, microbial growth, and flavor compound production (Liu et al., 2020). Energy metabolism accounted for 6.80%, while lipid metabolism and xenobiotics biodegradation and metabolism contributed 2.70% and 2.59%, respectively. Glycan biosynthesis and metabolism (3.55%) and metabolism of cofactors and vitamins (6.52%) also had significant contributions. The significant roles of energy metabolism, lipid metabolism, and xenobiotics biodegradation further highlight the microbial community's ability to adapt to the acidic and oxidative conditions characteristic of vinegar fermentation. Additionally, contributions from glycan biosynthesis and the metabolism of cofactors and vitamins reflect the metabolic versatility required for maintaining functionality throughout the fermentation process.

At Level 2, carbohydrate metabolism pathways were the most abundant, including fructose and mannose metabolism (5.08%), pyruvate metabolism (4.78%), and glycolysis/gluconeogenesis (4.07%) (Figure 3). These pathways play a central role in converting sugars into intermediates for acetic acid production. Within amino acid pathways, alanine, aspartate, and glutamate metabolism (3.24%) and glycine, serine, and threonine metabolism (5.32%) were prevalent. These pathways contributed significantly to microbial growth and the synthesis of flavor and aroma compounds (Pelicaen et al., 2019). Other relevant pathways within the Level 2 classification (e.g., oxidative phosphorylation, sulfur metabolism, unsaturated fatty acid biosynthesis, and benzoate degradation) demonstrated significant roles in supporting energy efficiency, microbial adaptation, and the processing of complex organic substrates, ensuring a clean and efficient fermentation matrix.

At Level 3, sub-pathways provided further detail (Figure 3). Within energy metabolism sub-pathways, oxidative phosphorylation, methane metabolism, and sulfur metabolism were prominently enriched. Several amino acid metabolism pathways (e.g., alanine, aspartate, glutamate, glycine, serine, and threonine) were reported, while pathways with a relative abundance of less than 1%, including certain lipid biosynthesis and secondary metabolite pathways, contributed to the system's metabolic versatility. These sub-pathway genes emphasize the importance of enriched amino acid and energy metabolism in supporting microbial growth, energy production, and adaptation, while less abundant genes highlight the metabolic versatility essential for natural vinegar fermentation (Kondakova et al., 2015).

Correlation between predominant species and predictive functions

The activities of *A. ghanensis, Leuc. pseudomesenteroides*, and *S. cerevisiae* were analyzed across critical metabolic pathways related to vinegar fermentation quality and the final product (Table 1). These pathways included amino acid biosynthesis, pyruvate metabolism, fructose and mannose metabolism, starch and sucrose metabolism, secondary metabolism, and the pentose phosphate pathway. The functional potential was classified based on the percentage of enzymes present within each metabolic pathway for the three major microbial groups, where “High” (≥80%) indicates a strong contribution, “Moderate” (10–79%) indicates a partial contribution, and “None” (<10%) indicates minimal or no involvement.

Table 1. Comparative analysis of metabolic pathway activities in *Acetobacter ghanensis, Leuconostoc pseudomesenteroides*, and *Saccharomyces cerevisiae* during natural apple fermentation.

Pathway/Metabolite	*Acetobacter ghanensis*	*Leuconostoc pseudomesenteroides*	*Saccharomyces cerevisiae*
Amino Acids			
Alanine	High	Moderate	High
Valine	High	Moderate	None
Leucine	High	Moderate	None
Isoleucine	Moderate	Moderate	None
Glutamate	Moderate	None	High
Proline	None	None	High
Lysine	None	None	Moderate
Phenylalanine	None	High	None
Tyrosine	None	High	None
Tryptophan	None	High	None
Methionine	Moderate	None	Moderate
Cysteine	Moderate	None	Moderate
Pyruvate Metabolism			
Acetyl-CoA Production	High	Moderate	High
Lactate Production	None	High	Moderate
Ethanol Production	None	None	High
Acetate Production	High	None	None
Oxaloacetate Formation	Moderate	None	Moderate
Formate Production	Moderate	None	None
Succinate Production	Moderate	None	None
Leucine/Isoleucine Synthesis	High	Moderate	None
Fructose and Mannose			
Fructose Utilization	High	Moderate	High
Mannose Utilization	High	High	Moderate
D-Mannitol Production[2]	High	High	None
Sorbitol Pathway	Moderate	High	None
Glycolysis Linkage	High	Moderate	High
L-Fucose Utilization[2]	None	Moderate	None
L-Rhamnose Utilization[2]	None	None	Moderate
Fructose-6P[1] Conversion	High	High	High
Starch and Sucrose			
Sucrose Utilization	High	High	High
Starch Degradation	Moderate	None	High
Maltose Utilization	None	None	High
Trehalose Metabolism	High	Moderate	High
Levan Biosynthesis	High	High	None
Inulin Utilization	Moderate	None	None
Cellobiose Utilization	High	None	None
Glucose-6P[1] Conversion	High	High	High
Secondary Metabolism			
Phenolic Compound Synthesis	High	Moderate	High
Terpenoid Biosynthesis	None	None	Moderate
Polyketide Biosynthesis	None	None	High
Alkaloid Synthesis	None	None	None
Non-ribosomal Peptide Biosynthesis	Moderate	None	High
Flavonoid Synthesis	High	Moderate	None

Isoprenoid Biosynthesis	Moderate	None	High
Steroid Biosynthesis	None	None	High
Pentose Phosphate Pathway			
Glucose-6P[1] Dehydrogenase	High	Moderate	High
Ribulose-5P[1] Epimerase	Moderate	High	High
Transketolase Activity	High	High	High
D-Ribose Synthesis[2]	High	Moderate	High
Sedoheptulose-7P[1] Synthesis	High	High	High
Nucleotide Sugar Biosynthesis	Moderate	Moderate	High

[1] P: Phosphate; [2] L- and D- prefixes denote the spatial arrangement of the hydroxyl groups.

In amino acid biosynthesis, *A. ghanensis* demonstrated high activity in the biosynthesis of alanine, valine, and leucine, which are precursors to branched-chain esters known for imparting fruity and sweet notes to vinegar (Es-Sbata et al., 2023; Pelicaen et al., 2019). *Leuc. pseudomesenteroides* exhibited high activity in aromatic amino acid pathways, including phenylalanine, tyrosine, and tryptophan. These aromatic amino acids are vital precursors for volatile aromatic compounds that significantly influence vinegar's sensory profile. For example, phenylalanine is converted into phenethyl alcohol, imparting floral notes, while tyrosine and tryptophan contribute to the formation of phenolic volatiles and indoles, which add complexity and depth to the aroma (Dzialo et al., 2017). Although *S. cerevisiae* exhibited limited activity in aromatic amino acid pathways, it played a pivotal role in ethanol production. During the fermentation process, ethanol acts as a precursor for the formation of esters and other volatile compounds that enhance aroma production (Dzialo et al., 2017).

In starch and sucrose metabolism, all three microorganisms showed high activity in sucrose utilization, emphasizing their role in breaking down disaccharides into fermentable sugars. *A. ghanensis* demonstrated high activity in trehalose metabolism, potentially enhancing the microbial tolerance to stress during fermentation (Hua et al., 2024) *Leuc. pseudomesenteroides*, with high activity in the sorbitol pathway, may

enhance the sweetness profile and act as a precursor for the synthesis of minor aromatic compounds (Dols et al., 1997).

S. cerevisiae complements this by facilitating glycolysis and mannose utilization (Table 1), maintaining metabolic balance and supporting overall fermentation efficiency. The integration of these pathways ensures a consistent conversion of sugars into flavor precursors. In secondary metabolism, *A. ghanensis* displayed high activity in phenolic compound synthesis and flavonoid biosynthesis. These processes contribute to the antioxidant properties of vinegar and enhance its health benefit (Zhao et al., 2021). *Leuc. pseudomesenteroides* played a moderate role in phenolic compound synthesis, with a potential function in stabilizing the final product.

The pentose phosphate pathway showed high activity across all three microorganisms. This activity supports the generation of precursors for secondary metabolites and aromatic compounds. For instance, ribulose-5P and sedoheptulose-7P are critical for synthesizing nucleotide sugars, which play a role in stabilizing the flavor and consistency of vinegar (Dringen et al., 2007). *S. cerevisiae* exhibited high activity in nucleotide sugar biosynthesis, further highlighting its role in enhancing the textural properties and flavor of the final product.

These findings highlight the complementary roles of AAB, LAB, and *Saccharomyces* yeast in vinegar fermentation. Their coordinated metabolic activities not only ensure efficient substrate conversion and production of essential compounds like acetic acid but also contribute to the formation of a diverse array of aroma and flavor compounds. This synergy results in a high-quality vinegar with a complex sensory profile, balancing acidity, sweetness, and aromatic richness while enhancing its nutritional and health-promoting properties.

Pyruvate metabolism and pectin degradation

We selected pyruvate metabolism and pectin degradation as focal pathways due to their critical roles in fermentation and their impact on product quality (Figure 4 and 5). Pyruvate metabolism links carbohydrate breakdown to key metabolites, while pectin degradation in apples releases sugars to drive microbial growth and efficiency (Muslu Can et al., 2024).

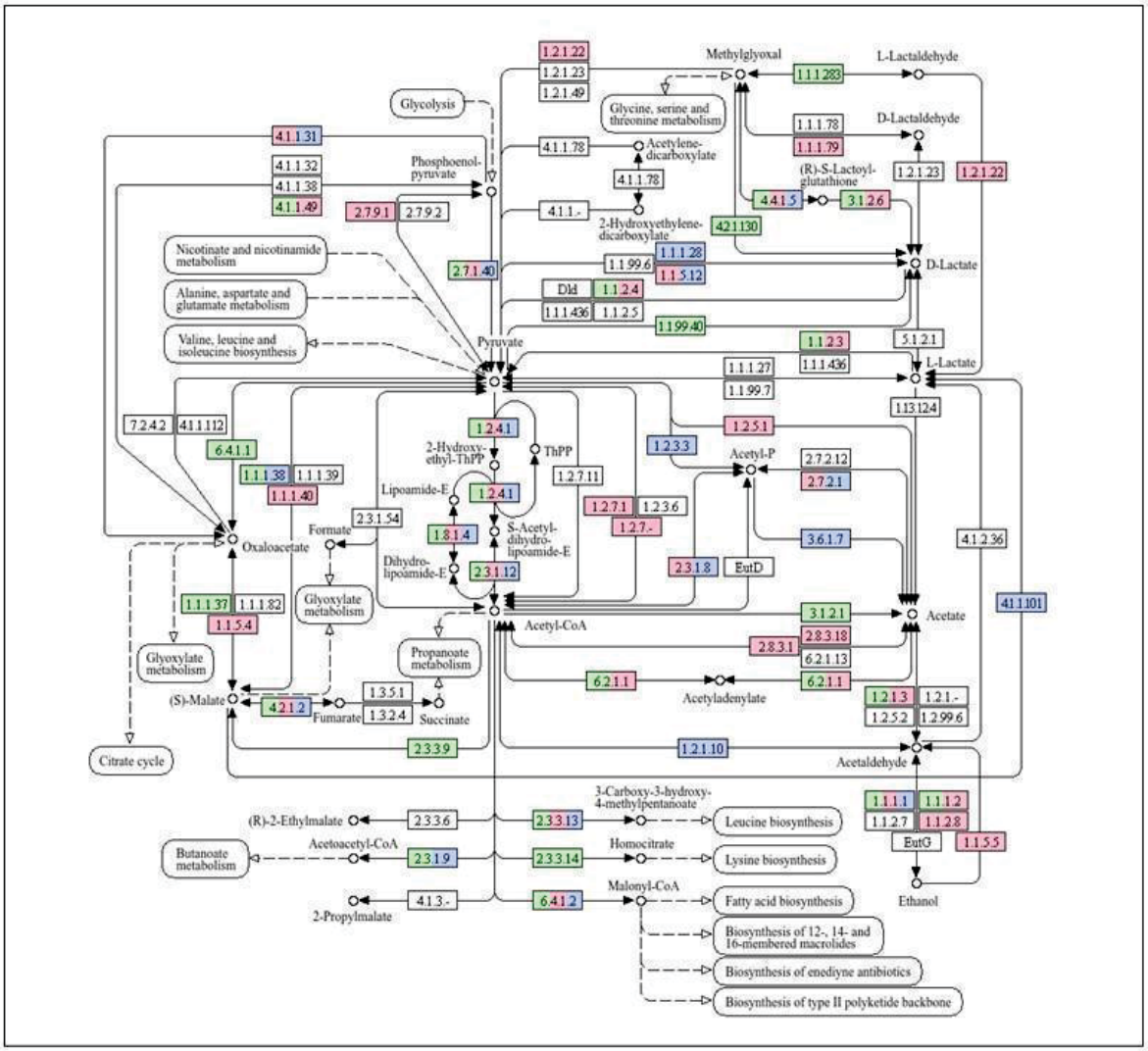


Figure 4. Pyruvate metabolism pathways involved in vinegar fermentation. Enzyme codes (EC numbers) are shown for each reaction step, with the contributing microorganisms indicated by color: *Saccharomyces cerevisiae* (green), *Acetobacter ghanensis* (pink), and *Leuconostoc pseudomesenteroides* (blue).

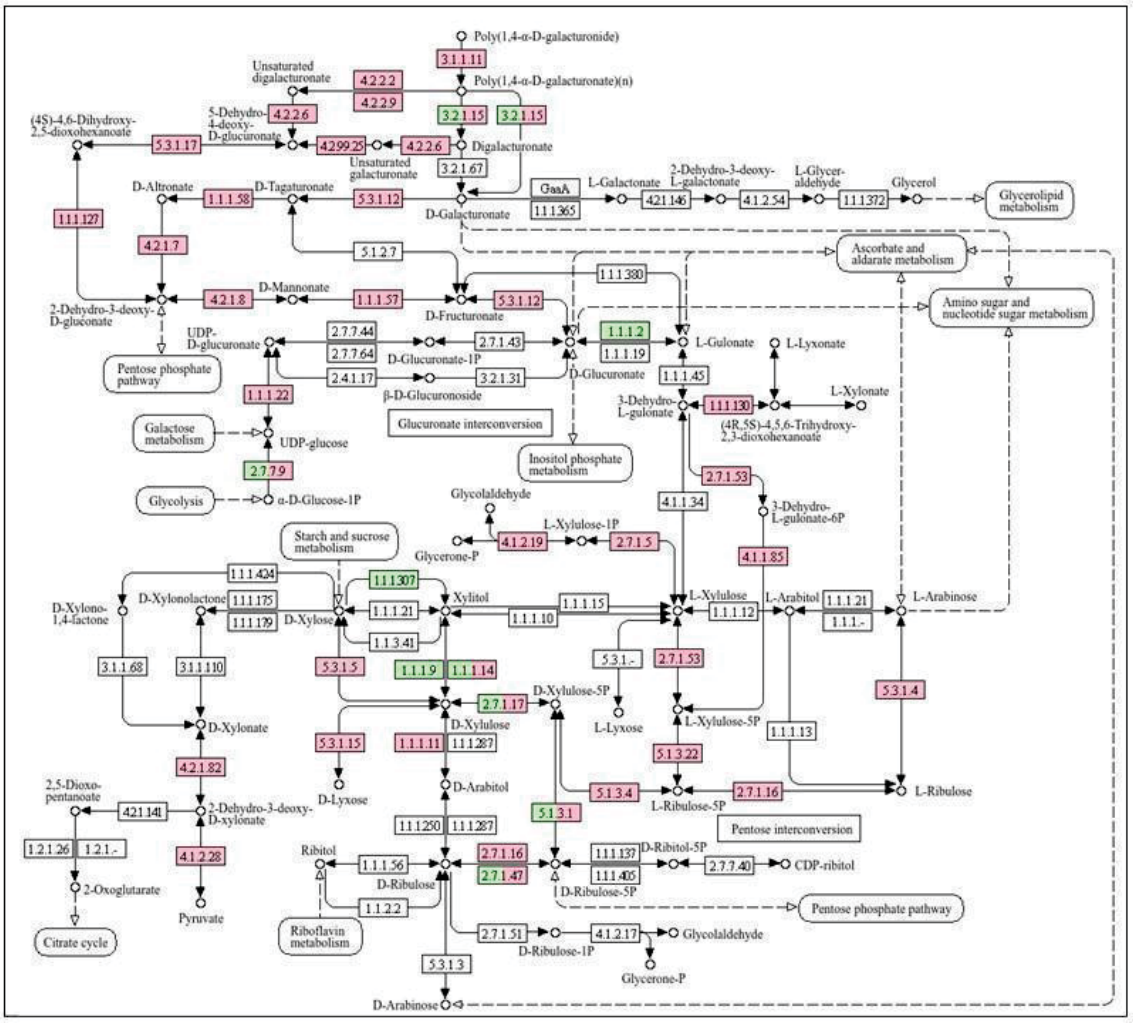


Figure 5. Pectin degradation (pentose and glucuronate interconversions) involved in vinegar fermentation. Enzyme codes (EC numbers) are shown for each reaction step, with the contributing microorganisms indicated by color: *Saccharomyces cerevisiae* (green), *Enterobacter* sp. (pink).

In *S. cerevisiae*, the active enzymes involved in pyruvate metabolism highlight its central role in ethanol production, particularly through the action of pyruvate decarboxylase (K01568) (Figure 4). Ethanol is a crucial substrate for acetic acid production in the oxidative processes carried out by AAB species and acts as a precursor for ester formation and aroma development in vinegar. Additionally, *S. cerevisiae* actively produces acetyl-CoA (K01895), a key intermediate for lipid biosynthesis and further ester production. Enzymes facilitating oxaloacetate formation, such as malate dehydrogenase (K00024) and phosphoenolpyruvate carboxykinase (PEPK, K01610),

suggest a secondary role in supporting amino acid biosynthesis, adding layers of flavor to the final product.

A. ghanensis demonstrated high activity in acetate and acetyl-CoA production, both of which are critical for acetic acid synthesis, the defining component of vinegar's sourness. Meanwhile, *Leuc. pseudomesenteroides* contributed significantly to lactate production, a key precursor for aroma compounds. For example, lactate can be converted into ethyl lactate, which enhances the vinegar's fruity and buttery notes (Özcan et al., 2019).

A. ghanensis showed active enzymes associated with acetic acid production through robust acetate and acetyl-CoA synthesis, primarily mediated by acetaldehyde dehydrogenase (ALDH, K00128) and acetyl-CoA synthetase (ACS, K01895) (Figure 4). These enzymes facilitate the conversion of acetaldehyde into acetyl-CoA, which is subsequently used in acetic acid synthesis, ensuring a high conversion rate of substrates and a clean fermentation process. Additionally, enzymes like cytochrome c oxidase (K02274) and NADH dehydrogenase (K00330) are critical in the electron transport chain. They drive oxidative phosphorylation, generating the energy required for these processes (Sprotte et al., 2021). The high enzymatic activity associated with oxidative metabolism in this pathway allows *A. ghanensis* to efficiently utilize energy while recycling metabolic byproducts, ensuring stability in the vinegar's acidity during the oxidative stages of fermentation. This activity directly contributes to its defining sourness and overall quality.

Leuc. pseudomesenteroides active enzymes contribute significantly to lactate production via lactate dehydrogenase (LDH, K00016) (Figure 4). Lactate serves as a precursor for compounds like ethyl lactate, which imparts fruity and buttery notes to the vinegar's aroma. Additionally, *Leuc. pseudomesenteroides* is involved in aromatic amino

acid metabolism, including pathways for phenylalanine (K00832) and tyrosine (K00830), utilizing enzymes like phenylalanine ammonia-lyase (PAL, K01595) and tyrosine aminotransferase (K00810). These pathways produce volatile compounds, such as phenethyl alcohol and 4-hydroxyphenylacetaldehyde, which enrich the sensory profile of the product (Özcan et al., 2019). This microorganism plays an essential role in balancing acidity with aromatic precursors.

Pectin, a complex polysaccharide found in apple cell walls, serves as an essential carbon source during fermentation. Composed of galacturonic acid and various sugar residues, its breakdown requires specific enzymes, with the efficiency of this process varying across microorganisms. Pectin, a complex polysaccharide found in apple cell walls, serves as an essential carbon source during fermentation. Composed of galacturonic acid and various sugar residues, its breakdown requires specific enzymes, with the efficiency of this process varying across microorganisms. Among the key players, *S. cerevisiae* demonstrated moderate activity, exhibiting polygalacturonase (EC 3.2.1.15) activity (Figure 5) but playing only a partial role in pectin degradation. In addition, *Leuc. pseudomesenteroides* and *Acetobacter ghanensis* showed no detectable pectinolytic activity, suggesting a limited role in pectin breakdown**.** Consequently, their ability to fully depolymerize pectin is limited, requiring the presence of additional microorganisms or enzymes produced by the fruit itself to achieve complete hydrolysis.

Given the high abundance of *Enterobacteriaceae* at the start of fermentation (Figure 1) and their known enzymatic activity (Abdollahzadeh et al., 2020; Lv et al., 2022; Vale et al., 2024), we focused on pentose and glucuronate interconversion in *Enterobacter* sp. (Figure 5). This microorganism showed strong potential for pectin degradation due to the presence of genes encoding key enzymes, including pectin lyase (EC 4.2.2.10), which breaks pectin into unsaturated digalacturonides; polygalacturonase

(EC 3.2.1.15), which hydrolyzes polygalacturonic acid; and pectinesterase (EC 3.1.1.11), which removes methoxy groups, facilitating further hydrolysis (Manyapu et al., 2022). In apple fermentation for vinegar production, this enzymatic activity promotes the breakdown of pectin into galacturonic acid and other fermentable monosaccharides, supporting glucuronate interconversion pathways and enhancing fermentation efficiency. However, the use of *Enterobacteriaceae* species requires caution due to risks such as undesirable metabolite production, competition with beneficial microorganisms, and safety concerns associated with certain strains (Mladenović et al., 2021). Balancing their enzymatic efficiency with proper control measures is crucial to maximizing benefits while ensuring fermentation quality and product safety.

Conclusions

This comprehensive study sheds light on microbial dynamics, bacteriophage interactions, and functional pathways that drive apple vinegar fermentation. We observed a rich microbial diversity throughout the fermentation process, including key vinegar-related species such as *A. ghanensis*, *Leuc. pseudomesenteroides*, and *S. cerevisiae*. The early stages were dominated by *Enterobacteriaceae*, followed by consecutive phases enriched in yeasts, LAB, and AAB. This succession is key to fermentation success, shaping the vinegar's acidity and flavor.

The virome analysis revealed the presence of bacteriophages, particularly those targeting spoilage bacteria like *Pseudomonas* and *Erwinia*, indicating a regulatory role in maintaining microbial stability. These findings underscore the potential of using bacteriophages to enhance fermentation control and product consistency.

From a functional perspective, the metagenomic analysis highlighted the critical roles of carbohydrate and amino acid metabolism, energy production, and glycan biosynthesis in supporting microbial growth and the production of key metabolites, including acetic acid, ethanol, and aromatic compounds. The significant contributions of

A. ghanensis, *Leuc. pseudomesenteroides*, and *S. cerevisiae* in these metabolic pathways were evident in their specific enzymatic activities, which directly influence the vinegar's sensory profile and health benefits.

In addition to providing new insights into microbial and viral interactions in vinegar fermentation, this study highlights the importance of optimizing microbial consortia and phage dynamics. These findings offer practical applications for improving the quality, stability, and nutritional value of vinegar, paving the way for future innovations in fermentation biotechnology.

FINAL CONCLUSIONS AND FUTURE PERSPECTIVES

This thesis demonstrated the complexity and significance of microbial and viral interactions in fermented foods, using natural vinegar fermentation as a model. By combining metagenomic, metabolomic, and viromic analyses, it uncovered critical insights into microbial succession, metabolic activity, and the influence of viruses on fermentation dynamics and product functionality. The findings emphasize the pivotal role of lactic acid bacteria and acetic acid bacteria in shaping the sensory and functional properties of vinegar, as well as the emerging importance of viruses in modulating microbial communities and enhancing fermentation processes.

Future research should focus on further elucidating the mechanisms underlying bacteria-virus interactions and their impact on fermentation efficiency and product quality. Expanding the application of advanced omics tools, such as transcriptomics and proteomics, can provide deeper insights into microbial metabolic pathways and gene functions. Additionally, the integration of synthetic biology approaches to engineer microbial consortia offers promising opportunities to optimize fermentation processes and develop novel functional foods. These efforts will contribute to advancing food biotechnology, ensuring sustainability, and meeting the growing consumer demand for safe, high-quality, and health-promoting fermented products.

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ANNEXES

Table 1S. Relative abundance (%) of bacteria and fungi identified during the fermentation of Brazilian apple vinegar.

Genus	Weeks								
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Bacteria									
Acetobacter	0	0	0.014133 8	0.053445 1	0.329004 3	0.308768 7	5.017131 2	82.72657 8	78.99257 5
Komagataeibacter	0	0	0	0	0.006568 1	5.362656 6	5.788535 1	0.513492 9	15.23864 4
Leuconostoc	0.280668 5	62.58983	62.0837	61.09719 7	47.59516 3	43.81689 3	71.08966 9	12.74109	2.505436 1
Oenococcus	0	0	0.014604 9	0.158239 5	0.896253 2	2.442952 4	2.551500 9	1.865071 3	1.894771 7
Gluconobacter	0.115250 3	19.19205 2	22.35544 6	22.79958 1	27.44469 3	25.08652 5	10.17423 1	0.990580 4	0.509647 9
Tatumella	0.020338 3	4.787451 5	4.566633 8	5.355514 8	9.123749 8	9.148939 6	1.682490 7	0.309317 7	0.094738 6
Novosphingobium	1.978916	0	0.013191 6	0	0.021495 7	0.019198 1	0.024616 2	0.028513 2	0.033101 4
Lelliottia	0.061692 8	0.888987 8	1.163683 6	1.296306	2.369308 9	2.821580 7	0.948011 1	0.126527 5	0.021116 4
Rahnella	0.189146 1	3.501894 3	2.707566 8	2.442232 1	3.542618 3	3.179944 4	0.606532 5	0.095468 4	0.017692 1
Klebsiella	0	1.395034	2.048460 1	1.746397 7	3.180773 2	3.546307 3	0.822640 1	0.106924 6	0.011414 3
Pseudomonas	3.052099	0.029981	0.023085	0.013099	0.002388	0	0.006583	0	0.003995

	9	2	2	3	4		4		
	24.57204	0.083583	0.036276	0.041393	0.060307	0.041062	0.002289		0.001141
Luteibacter	8	9	8	8	5	5	9	0	4
							0.020322	0.046588	
Methylophilus	0	0	0	0	0	0	7	6	0
							0.015456	0.034114	
Methylotenera	0	0	0	0	0	0	7	1	0
	14.72695	5.521990	3.479272	3.197275	3.866845	2.959166	0.236430	0.024185	
Erwinia	8	8	8	3	8	8	3	3	0
	29.23358	0.551926	0.407053		0.074040				
Cronobacter	5	5	7	0.667016	9	0	0	0	0
	2.341615	0.012719	0.002826	0.011003			0.024616		
Sphingomonas	5	3	8	4	0	0	2	0.023167	0
	0.565404							0.006619	
Curtobacterium	6	0	0	0	0	0	0	1	0
	2.778888	0.002725		0.001047			0.000572		
Methylobacterium	9	6	0	9	0	0	5	0	0
	0.650825	0.002725	0.004240		0.002388	0.034663			
Xanthomonas	4	6	1	0	4	2	0	0	0
	7.931934	0.096303	0.061246	0.077023	0.007165				
Agrobacterium	5	2	5	8	2	0	0	0	0
	0.671163	0.007722		0.011527					
Lactococcus	7	4	0.003769	4	0	0	0	0	0
	0.865055	0.008630		0.005239					
Pseudoxanthomonas	4	9	0	7	0	0	0	0	0
	0.660994		0.002826	0.003143					
Rhizobium	5	0	8	8	0	0	0	0	0
Neorhizobium	1.566726	0.005451	0	0	0	0	0	0	0

Endobacter	6 0.857598	1 0	0	0	0	0	0	0	0
Lichenicola	0.665740 1	0	0	0	0	0	0	0	0
Ralstonia	0	0	0	0	0	0	0.019464	0.022912 4	0.376100 8
Burkholderia	0	0	0.003297 9	0	0.005971	0.013332	0.048946 2	0.021639 5	0.130693 6
Achromobacter	0.341005 4	0.001817	0.002355 6	0	0	0	0.041504 1	0.033095 7	0.055359 3
Serratia	0	0	0	0	0	0	0.107910 7	0.093177 2	0.049652 1
Oryzomicrobium	0	0	0	0	0	0	0.004579 8	0.00611	0.018262 9
Phyllobacterium	0	0	0	0	0	0	0.004293 5	0.003818 7	0.00799
Faecalibacterium	0.035253	0.019987 5	0.000942 3	0.029866 4	0.034034 9	0	0.021467 6	0.022912 4	0.006848 6
Streptomyces	0	0	0	0	0	0.013865 3	0.001431 2	0.000763 7	0.006277 9
Comamonas	0	0	0	0	0	0	0.002576 1	0.004837 1	0.006277 9
Genero	0.000677 9	0.000908 5	0.001413 4	0.002095 9	0.002985 5	0.003199 7	0.002003 6	0.002036 7	0.005707 1
Bacteroides	0	0	0	0.003143 8	0	0	0.004293 5	0.007128 3	0.005707 1
Herbaspirillum	0.108470 9	0	0	0	0	0	0	0	0.003995

							0.002862		0.001712
Enterenecus	0	0	0	0	0	0	4	0	1
									0.001141
Schleiferia	0	0	0	0	0	0	0	0	4
	0.002711								
Acinetobacter	8	0	0	0	0	0	0	0	0
		0.006813							
Enterobacter	0	9	0	0	0	0	0	0	0
Polynucleobacter	0	0	0	0	0	0	0	0	0
Methylopumilus	0	0	0	0	0	0	0	0	0
	0.025083						0.001144		
Corynebacterium	9	0	0	0	0	0	9	0	0
							0.003434		
Enterococcus	0	0	0	0	0	0	8	0	0
	0.045422	0.040429		0.008907	0.120017	0.193580	0.005724	0.007892	
Lactobacillus	2	2	0.051824	5	9	4	7	1	0
	0.067794		0.002826		0.021495		0.008873		
Staphylococcus	3	0	8	0	7	0	3	0	0
Agathobaculum	0	0	0	0	0	0	0	0	0
Limnobacter	0	0	0	0	0	0	0	0	0
						0.002133			
Nitrospira	0	0	0	0	0	1	0	0	0
	0.014914				0.059113				
Bradyrhizobium	7	0	0	0	3	0	0	0	0
	0.005423	0.003634		0.006287			0.004579		
Agathobacter	5	1	0	7	0	0	8	0	0
Methylocystis	0	0	0	0	0	0	0	0	0
Liquorilactobacillus	0	0.015899	0.055121	0.082787	0.037020	0.039995	0.024043	0.008146	0

		1	9	5	5	9	8	6	
	0.073895	0.000908	0.000942						
Pedobacter	8	5	3	0	0	0	0	0	0
Solibacillus	0	0	0	0	0	0	0	0	0
Succinivibrio	0	0	0	0	0	0	0	0	0
Pseudonocardia	0	0	0	0	0	0	0	0	0
	0.006101					0.007999	0.009159	0.044806	
Bacillus	5	0	0	0	0	2	5	5	0
							0.015170	0.012983	
Cyanobium	0	0	0	0	0	0	5	7	0
		0.230310	0.192219	0.146188		0.252774	0.044080	0.011456	
Pantoea	0.250161	1	8	1	0.189282	4	2	2	0
							0.024043	0.010437	
Clostridium	0	0	0	0	0	0	8	9	0
	0.410155	0.002725					0.002003	0.009419	
Flavobacterium	6	6	0	0	0	0	6	6	0
								0.007382	
Mediterraneibacter	0	0	0	0	0	0.026664	0	9	0
	0.092878	0.021350		0.041917	0.016121		0.012594	0.005600	
Gemmiger	2	2	0	7	8	0.022931	4	8	0
	0.288803		0.008009	0.030390	0.108075	0.002666	0.003721	0.005091	
Phocaeicola	8	0.022713	2	4	8	4	1	6	0
	0.013558							0.002800	
Escherichia	9	0	0	0	0	0	0	4	0
								0.002545	
Zoogloea	0	0	0	0	0	0	0	8	0
	0.033897							0.002291	
Stenotrophomonas	2	0	0	0	0	0	0	2	0

Faecousia	0	0	0	0	0	0	0.002003 6	0.001782 1	0
Terrisporobacter	0	0	0	0	0	0	0.003434 8	0.001527 5	0
Limosilactobacillus	0.022372 1	0.075861 5	0.038632 4	0.011003 4	0.077623 5	0.054394 5	0	0.001527 5	0
Fimimorpha	0	0	0	0	0	0	0	0.001527 5	0
Eubacterium	0.008813 3	0	0	0	0	0	0.002003 6	0.001272 9	0
Anaerofilum	0.001355 9	0	0	0	0	0	0	0.001272 9	0
Caproicibacterium	0	0	0	0	0	0	0	0.001272 9	0
Lawsonibacter	0	0	0	0	0	0	0	0.001272 9	0
Anaerotruncus	0	0	0	0	0	0	0	0.001018 3	0
Sharpea	0	0	0	0	0	0	0.222118 5	0	0
Sodaliphilus	0.004067 7	0	0	0	0	0	0.046370 1	0	0
Phytobacter	0.09559	0.484241 7	0.447570 6	0.371495 9	0.398268 4	0.164783 3	0.040359 2	0	0
Bulleidia	0	0	0	0	0	0	0.038641 8	0	0
Ruminococcus	0.010169 1	0.007268 2	0	0	0	0	0.031485 9	0	0

Holdemanella	0.089488 5	0.002271 3	0	0.006287 7	0.005971	0.089057 6	0.026047 4	0	0
Citrobacter	0	0.215319 5	0.153116 3	0.156143 6	0.173160 2	0.138652 6	0.025474 9	0	0
Intestinibacter	0	0	0	0	0	0	0.013739 3	0	0
Intestinimonas	0	0	0	0	0	0	0.012308 1	0	0
Prevotella	0.012203	0.024984 3	0	0.026722 6	0	0	0.011735 6	0	0
Parafannyhessea	0	0	0	0	0	0	0.011735 6	0	0
Absicoccus	0	0	0	0	0	0	0.009732	0	0
Alloprevotella	0.002711 8	0	0	0	0	0	0.009445 8	0	0
Blautia	0.014236 8	0	0	0.003667 8	0.006568 1	0.007999 2	0.008300 8	0	0
Copromorpha	0	0	0	0	0	0	0.006010 9	0	0
Ruthenibacterium	0	0	0	0	0	0	0.005438 5	0	0
Cupriavidus	0	0	0.000942 3	0	0	0.001066 6	0.005152 2	0	0
Sphingomicrobium	0.098301 8	0	0	0.004715 7	0	0	0.004007 3	0	0
Lachnospira	0	0.001817	0	0	0	0	0.003721 1	0	0
Paratractidigestivibacter	0	0	0	0	0	0	0.003434	0	0

							8		
		0.000908					0.002862		
Parabacteroides	0	5	0	0	0	0	4	0	0
							0.002576		
Senegalimassilia	0	0	0	0	0	0	1	0	0
							0.002289		
Caproicibacter	0	0	0	0	0	0	9	0	0
							0.002289		
Emergencia	0	0	0	0	0	0	9	0	0
	0.003389	0.002725		0.007859	0.004179		0.002003		
Collinsella	7	6	0	6	7	0	6	0	0
							0.002003		
Hyphomicrobium	0	0	0	0	0	0	6	0	0
							0.001717		
Dorea	0	0	0	0	0	0	4	0	0
		0.000908					0.001431		
Microvirga	0	5	0	0	0.057322	0	2	0	0
							0.001431		
Fimenecus	0	0	0	0	0	0	2	0	0
							0.001144		
Planktophila	0	0	0	0	0	0	9	0	0
							0.001144		
Reyranella	0	0	0	0	0	0	9	0	0
					0.001791		0.000858		
Cryptobacteroides	0	0	0	0	3	0	7	0	0
				0.003667			0.000858		
Phascolarctobacterium	0	0	0	8	0	0	7	0	0
Butyricicoccus	0.002711	0	0	0	0	0	0.000858	0	0

	8						7		
							0.000858		
Merdicola	0	0	0	0	0	0	7	0	0
							0.000858		
Tumebacillus	0	0	0	0	0	0	7	0	0
							0.000572		
Onthovivens	0	0	0	0	0	0	5	0	0
	0.023050	0.014536	0.016489			0.051194			
Asaia	1	3	4	0	0	8	0	0	0
						0.034663			
Oryzihumus	0	0	0	0	0	2	0	0	0
						0.028797			
Salinicoccus	0	0	0	0	0	1	0	0	0
	0.006101	0.046788	0.029209	0.017291	0.020301	0.020264			
Ligilactobacillus	5	8	9	1	5	6	0	0	0
	0.019660			0.005239		0.011732			
Streptococcus	4	0	0	7	0	1	0	0	0
	0.010169			0.005239		0.009065			
Massilia	1	0	0	7	0	7	0	0	0
						0.006399			
Cutibacterium	0	0	0	0	0	4	0	0	0
						0.006399			
Lachnoclostridium	0	0	0	0	0	4	0	0	0
	0.020338								
Mycobacterium	3	0	0	0	0	0.003733	0	0	0
						0.003199			
Spirillospora	0	0	0	0	0	7	0	0	0
Bifidobacterium	0.021016	0.002271	0	0.009431	0	0.002666	0	0	0

	2	3		5		4			
		0.003179				0.002666			
Moraxella	0	8	0	0	0	4	0	0	0
						0.002666			
Ktedonobacter	0	0	0	0	0	4	0	0	0
			0.001884		0.008359	0.002133			
Sulfurimonas	0	0	5	0	5	1	0	0	0
						0.002133			
Kitasatospora	0	0	0	0	0	1	0	0	0
						0.002133			
Noviherbaspirillum	0	0	0	0	0	1	0	0	0
						0.002133			
Paludibaculum	0	0	0	0	0	1	0	0	0
				0.001571		0.001599			
Solirubrobacter	0	0	0	9	0	8	0	0	0
						0.001599			
Dormibacter	0	0	0	0	0	8	0	0	0
						0.001066			
Rubrimentiphilum	0	0	0	0	0	6	0	0	0
					0.057919				
Yaniella	0	0	0	0	1	0	0	0	0
					0.043588				
Blastococcus	0	0	0	0	6	0	0	0	0
	0.002711				0.008956				
Alistipes	8	0	0	0	6	0	0	0	0
Niallia	0	0	0	0	0.005971	0	0	0	0
					0.002985				
Erysipelatoclostridium	0	0	0	0	5	0	0	0	0

Rubneribacter	0	0	0	0	0.002985 5	0	0	0	0
Acidithrix	0	0	0	0	0.002388 4	0	0	0	0
Sulfotelmatobacter	0	0	0	0	0.001791 3	0	0	0	0
Trebonia	0	0	0	0	0.001791 3	0	0	0	0
Udaeobacter	0	0	0	0	0.001194 2	0	0	0	0
Fusobacterium	0	0.013627 8	0	0.008383 5	0	0	0	0	0
Porphyromonas	0.020338 3	0.005451 1	0	0.005763 7	0	0	0	0	0
Bergeyella	0.006779 4	0.003634 1	0	0.005239 7	0	0	0	0	0
Barnesiella	0	0	0	0.004715 7	0	0	0	0	0
Jatrophihabitans	0.292871 4	0	0	0.003667 8	0	0	0	0	0
Adlercreutzia	0.006779 4	0.008176 7	0	0.003143 8	0	0	0	0	0
Aureimonas	0.002711 8	0	0	0.002619 9	0	0	0	0	0
Nocardioides	0.050845 7	0	0	0.002095 9	0	0	0	0	0
Slackia	0	0	0	0.002095 9	0	0	0	0	0

Gordonibacter	0	0	0	0.001571 9	0	0	0	0	0
Amycolatopsis	0	0	0	0.001047 9	0	0	0	0	0
Christensenella	0	0	0	0.001047 9	0	0	0	0	0
Metallibacterium	0	0	0.001884 5	0	0	0	0	0	0
Humisphaera	0.007457 4	0	0.001413 4	0	0	0	0	0	0
Acidulodesulfobacterium	0	0	0.000942 3	0	0	0	0	0	0
Patulibacter	0	0	0.000942 3	0	0	0	0	0	0
Paracoccus	0	0.007268 2	0	0	0	0	0	0	0
Trichococcus	0	0.006813 9	0	0	0	0	0	0	0
Romboutsia	0	0.005905 4	0	0	0	0	0	0	0
Frederiksenia	0	0.002271 3	0	0	0	0	0	0	0
PMG-095	0	0.002271 3	0	0	0	0	0	0	0
Scatomonas	0	0.002271 3	0	0	0	0	0	0	0
Desulfovibrio	0.002033 8	0.001817	0	0	0	0	0	0	0

Oceanivirga	0.0020338	0.001817	0	0	0	0	0	0	0
Methyloceanibacter	0.0013559	0.0013628	0	0	0	0	0	0	0
Desulfofustis	0	0.0013628	0	0	0	0	0	0	0
Oribacterium	0	0.0013628	0	0	0	0	0	0	0
Phycicoccus	0	0.0013628	0	0	0	0	0	0	0
Terriglobus	0.0366089	0.0009085	0	0	0	0	0	0	0
Bruticola	0.0020338	0.0009085	0	0	0	0	0	0	0
Humimicrobium	0	0.0009085	0	0	0	0	0	0	0
Veillonella	0	0.0009085	0	0	0	0	0	0	0
Frondihabitans	0.2949053	0	0	0	0	0	0	0	0
Pigmentiphaga	0.2928714	0	0	0	0	0	0	0	0
Duganella	0.2454154	0	0	0	0	0	0	0	0
Paraburkholderia	0.2271109	0	0	0	0	0	0	0	0
Frankia	0.2196536	0	0	0	0	0	0	0	0

Caballeronia	0.183722 6	0	0	0	0	0	0	0	0
Tardiphaga	0.170163 7	0	0	0	0	0	0	0	0
Pararhizobium	0.150503 4	0	0	0	0	0	0	0	0
Rhizorhabdus	0.138300 4	0	0	0	0	0	0	0	0
Kineococcus	0.105081 2	0	0	0	0	0	0	0	0
Aquisphaera	0.101691 5	0	0	0	0	0	0	0	0
Roseomonas	0.09559	0	0	0	0	0	0	0	0
Bosea	0.094234 1	0	0	0	0	0	0	0	0
Variovorax	0.092200 3	0	0	0	0	0	0	0	0
Fibrobacter	0.088132 6	0	0	0	0	0	0	0	0
Janthinobacterium	0.084742 9	0	0	0	0	0	0	0	0
Spirosoma	0.079997 3	0	0	0	0	0	0	0	0
Subtercola	0.075251 7	0	0	0	0	0	0	0	0
Beijerinckia	0.073217 9	0	0	0	0	0	0	0	0
Mucilaginibacter	0.073217	0	0	0	0	0	0	0	0

	9								
	0.072539								
Brevundimonas	9	0	0	0	0	0	0	0	0
	0.068472								
Yonghaparkia	3	0	0	0	0	0	0	0	0
	0.066438								
Rubrivivax	4	0	0	0	0	0	0	0	0
	0.051523								
Azospirillum	7	0	0	0	0	0	0	0	0
	0.039320								
Fimbriimonas	7	0	0	0	0	0	0	0	0
	0.031863								
Finegoldia	3	0	0	0	0	0	0	0	0
	0.031185								
Rhizomicrobium	4	0	0	0	0	0	0	0	0
	0.020338								
Faecalibacillus	3	0	0	0	0	0	0	0	0
	0.019660								
Chryseobacterium	4	0	0	0	0	0	0	0	0
	0.018982								
Clavibacter	4	0	0	0	0	0	0	0	0
	0.014914								
Williamsia	7	0	0	0	0	0	0	0	0
	0.014236								
Aurantimonas	8	0	0	0	0	0	0	0	0
	0.014236								
Sphingobium	8	0	0	0	0	0	0	0	0
Granulicella	0.012203	0	0	0	0	0	0	0	0

Onthenecus	0.012203	0	0	0	0	0	0	0	0
Hymenobacter	0.010847 1	0	0	0	0	0	0	0	0
Mesorhizobium	0.010169 1	0	0	0	0	0	0	0	0
Acidisoma	0.008813 3	0	0	0	0	0	0	0	0
Rhodococcus	0.008813 3	0	0	0	0	0	0	0	0
Rhizobacter	0.008135 3	0	0	0	0	0	0	0	0
Hansschlegelia	0.006779 4	0	0	0	0	0	0	0	0
Granulicatella	0.006101 5	0	0	0	0	0	0	0	0
Allobaculum	0.005423 5	0	0	0	0	0	0	0	0
Alsobacter	0.005423 5	0	0	0	0	0	0	0	0
Luteimonas	0.005423 5	0	0	0	0	0	0	0	0
Paraclostridium	0.005423 5	0	0	0	0	0	0	0	0
Aeromicrobium	0.004745 6	0	0	0	0	0	0	0	0
Kineobactrum	0.004745 6	0	0	0	0	0	0	0	0
Limivicinus	0.004745	0	0	0	0	0	0	0	0

	6								
	0.004745								
Megasphaera	6	0	0	0	0	0	0	0	0
	0.004745								
Micrococcus	6	0	0	0	0	0	0	0	0
	0.003389								
Salipiger	7	0	0	0	0	0	0	0	0
	0.003389								
Sphingobacterium	7	0	0	0	0	0	0	0	0
	0.002711								
Helcococcus	8	0	0	0	0	0	0	0	0
	0.002711								
Kaistia	8	0	0	0	0	0	0	0	0
	0.002033								
Oceanimonas	8	0	0	0	0	0	0	0	0
	0.002033								
Perlucidibaca	8	0	0	0	0	0	0	0	0
	0.001355								
Arcanobacterium	9	0	0	0	0	0	0	0	0
Fungi									
	18.77323	96.47373	97.76563	98.31334	89.84802	64.76185	24.39878	2.823772	77.69515
Saccharomyces	4	5	9	6	6	2	9	9	1
	10.54832	0.009980	0.025274	0.005405	0.234811		16.57032	67.31224	2.603075
Pichia	7	9	7	9	4	17.52541	1	1	8
	2.416356	0.003659		0.001138				0.994234	
Candida	9	7	0	1	0	0	0	2	0
	14.68401	0.032604	0.020382	0.007113		0.029907			
Yarrowia	5	4	8	1	0.005135	6	0	0	0.016013

Kluyveromyces	9.526022 3	0.015304 1	0	0	0	0.009505 5	0	0	0
Hanseniaspora	0	3.213861 5	1.990990 8	0.928115 2	0.710269 4	3.933897 2	3.427740 8	2.757244 2	0.628991 2
Leotiomycetes	15.52044 6	0.012309 8	0	0.000853 6	0	0.018779 2	0	0	0
Alternaria	12.22119	0.043250 7	0.006522 5	0.003414 3	0.001400 5	0	0	0	0
Brettanomyces	0	0	0.023236 4	0.645014 4	9.126833 2	13.50967 2	55.57456 1	22.16513 9	18.96773 7
Cladosporium	3.763940 5	0.003992 4	0.004076 6	0.002560 7	0	0	0	0.443524 5	0
Sistotrema	2.416356 9	0.010646 3	0.001630 6	0.001707 1	0	0	0	0	0
Helotiales	2.276951 7	0	0	0	0	0	0	0	0
Boeremia	1.765799 3	0.005988 6	0	0	0	0	0	0	0
Sympodiomycopsis	1.16171	0	0	0	0	0	0	0	0
Aspergillus	1.115241 6	0.005988 6	0	0	0	0	0.022289 5	0	0
Bullera	1.022304 8	0	0.004076 6	0	0	0	0	0	0
Vishniacozyma	0.882899 6	0	0	0	0	0	0	0	0
Clavispora	0.697026	0.005988 6	0	0.001138 1	0	0	0	0	0
Papiliotrema	0.650557	0.007984	0	0.001422	0	0	0	0	0

	6	7		6					
	0.557620							0.543317	
Ophiosphaerella	8	0	0	0	0	0	0	6	0
								0.358515	
Agaricomycetes	0	0	0	0	0	0	0	7	0
								0.247634	
Debaryomyces	0	0	0	0	0	0	0	5	0
								1.644736	
Exobasidium	0	0	0	0	0	0	0	8	0
		0.121434	0.151647	0.083934	0.049249	0.210976	0.006299		
Kurtzmaniella	0	7	9	4	7	3	2	0	0.016013
			0.002038						
Malassezia	0	0	3	0	0	0	0	0	0
					0.024274			0.709639	
Malassezia	0	0	0	0	7	0	0	3	0
				0.001422					0.018575
Penicillium	0	0	0	6	0	0	0	0	1
		0.002994							
Rhodotorula	0	3	0	0	0	0	0	0	0
		0.010313							
Sporobolomyces	0	6	0	0	0	0	0	0	0
		0.001996	0.004484	0.001991					0.054444
Starmerella	0	2	2	7	0	0	0	0	3
		0.003659		0.001422					
Tetracladium	0	7	0	6	0	0	0	0	0
Yamadazyma	0	0.014306	0	0	0	0	0	0	0

Table 1S. Relative abundance (%) of bacteria and fungi identified during the fermentation of Brazilian apple cider vinegar.

Genus	Weeks								
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Bacteria									
Acetobacter	0	0	0.014133 8	0.053445 1	0.329004 3	0.308768 7	5.017131 2	82.72657 8	78.99257 5
Komagataeibacter	0	0	0	0	0.006568 1	5.362656 6	5.788535 1	0.513492 9	15.23864 4
Leuconostoc	0.280668 5	62.58983	62.0837	61.09719 7	47.59516 3	43.81689 3	71.08966 9	12.74109	2.505436 1
Oenococcus	0	0	0.014604 9	0.158239 5	0.896253 2	2.442952 4	2.551500 9	1.865071 3	1.894771 7
Gluconobacter	0.115250 3	19.19205 2	22.35544 6	22.79958 1	27.44469 3	25.08652 5	10.17423 1	0.990580 4	0.509647 9
Tatumella	0.020338 3	4.787451 5	4.566633 8	5.355514 8	9.123749 8	9.148939 6	1.682490 7	0.309317 7	0.094738 6
Novosphingobium	1.978916	0	0.013191 6	0	0.021495 7	0.019198 1	0.024616 2	0.028513 2	0.033101 4
Lelliottia	0.061692 8	0.888987 8	1.163683 6	1.296306	2.369308 9	2.821580 7	0.948011 1	0.126527 5	0.021116 4
Rahnella	0.189146 1	3.501894 3	2.707566 8	2.442232 1	3.542618 3	3.179944 4	0.606532 5	0.095468 4	0.017692 1
Klebsiella	0	1.395034	2.048460 1	1.746397 7	3.180773 2	3.546307 3	0.822640 1	0.106924 6	0.011414 3
Pseudomonas	3.052099 9	0.029981 2	0.023085 2	0.013099 3	0.002388 4	0	0.006583 4	0	0.003995
Luteibacter	24.57204	0.083583	0.036276	0.041393	0.060307	0.041062	0.002289	0	0.001141

	8	9	8	8	5	5	9		4
							0.020322	0.046588	
Methylophilus	0	0	0	0	0	0	7	6	0
							0.015456	0.034114	
Methylotenera	0	0	0	0	0	0	7	1	0
	14.72695	5.521990	3.479272	3.197275	3.866845	2.959166	0.236430	0.024185	
Erwinia	8	8	8	3	8	8	3	3	0
	29.23358	0.551926	0.407053		0.074040				
Cronobacter	5	5	7	0.667016	9	0	0	0	0
	2.341615	0.012719	0.002826	0.011003			0.024616		
Sphingomonas	5	3	8	4	0	0	2	0.023167	0
	0.565404							0.006619	
Curtobacterium	6	0	0	0	0	0	0	1	0
	2.778888	0.002725		0.001047			0.000572		
Methylobacterium	9	6	0	9	0	0	5	0	0
	0.650825	0.002725	0.004240		0.002388	0.034663			
Xanthomonas	4	6	1	0	4	2	0	0	0
	7.931934	0.096303	0.061246	0.077023	0.007165				
Agrobacterium	5	2	5	8	2	0	0	0	0
	0.671163	0.007722		0.011527					
Lactococcus	7	4	0.003769	4	0	0	0	0	0
	0.865055	0.008630		0.005239					
Pseudoxanthomonas	4	9	0	7	0	0	0	0	0
	0.660994		0.002826	0.003143					
Rhizobium	5	0	8	8	0	0	0	0	0
	1.566726	0.005451							
Neorhizobium	6	1	0	0	0	0	0	0	0
Endobacter	0.857598	0	0	0	0	0	0	0	0

Lichenicola	0.6657401	0	0	0	0	0	0	0	0
Ralstonia	0	0	0	0	0	0	0.019464	0.022912 4	0.376100 8
Burkholderia	0	0	0.003297 9	0	0.005971	0.013332	0.048946 2	0.021639 5	0.130693 6
Achromobacter	0.341005 4	0.001817	0.002355 6	0	0	0	0.041504 1	0.033095 7	0.055359 3
Serratia	0	0	0	0	0	0	0.107910 7	0.093177 2	0.049652 1
Oryzomicrobium	0	0	0	0	0	0	0.004579 8	0.00611	0.018262 9
Phyllobacterium	0	0	0	0	0	0	0.004293 5	0.003818 7	0.00799
Faecalibacterium	0.035253	0.019987 5	0.000942 3	0.029866 4	0.034034 9	0	0.021467 6	0.022912 4	0.006848 6
Streptomyces	0	0	0	0	0	0.013865 3	0.001431 2	0.000763 7	0.006277 9
Comamonas	0	0	0	0	0	0	0.002576 1	0.004837 1	0.006277 9
Genero	0.000677 9	0.000908 5	0.001413 4	0.002095 9	0.002985 5	0.003199 7	0.002003 6	0.002036 7	0.005707 1
Bacteroides	0	0	0	0.003143 8	0	0	0.004293 5	0.007128 3	0.005707 1
Herbaspirillum	0.108470 9	0	0	0	0	0	0	0	0.003995
Enterenecus	0	0	0	0	0	0	0.002862 4	0	0.001712 1

									0.001141
Schleiferia	0	0	0	0	0	0	0	0	4
	0.002711								
Acinetobacter	8	0	0	0	0	0	0	0	0
		0.006813							
Enterobacter	0	9	0	0	0	0	0	0	0
Polynucleobacter	0	0	0	0	0	0	0	0	0
Methylopumilus	0	0	0	0	0	0	0	0	0
	0.025083						0.001144		
Corynebacterium	9	0	0	0	0	0	9	0	0
							0.003434		
Enterococcus	0	0	0	0	0	0	8	0	0
	0.045422	0.040429		0.008907	0.120017	0.193580	0.005724	0.007892	
Lactobacillus	2	2	0.051824	5	9	4	7	1	0
	0.067794		0.002826		0.021495		0.008873		
Staphylococcus	3	0	8	0	7	0	3	0	0
Agathobaculum	0	0	0	0	0	0	0	0	0
Limnobacter	0	0	0	0	0	0	0	0	0
						0.002133			
Nitrospira	0	0	0	0	0	1	0	0	0
	0.014914				0.059113				
Bradyrhizobium	7	0	0	0	3	0	0	0	0
	0.005423	0.003634		0.006287			0.004579		
Agathobacter	5	1	0	7	0	0	8	0	0
Methylocystis	0	0	0	0	0	0	0	0	0
		0.015899	0.055121	0.082787	0.037020	0.039995	0.024043	0.008146	
Liquorilactobacillus	0	1	9	5	5	9	8	6	0
Pedobacter	0.073895	0.000908	0.000942	0	0	0	0	0	0

	8	5	3						
Solibacillus	0	0	0	0	0	0	0	0	0
Succinivibrio	0	0	0	0	0	0	0	0	0
Pseudonocardia	0	0	0	0	0	0	0	0	0
Bacillus	0.006101 5	0	0	0	0	0.007999 2	0.009159 5	0.044806 5	0
Cyanobium	0	0	0	0	0	0	0.015170 5	0.012983 7	0
Pantoea	0.250161	0.230310 1	0.192219 8	0.146188 1	0.189282	0.252774 4	0.044080 2	0.011456 2	0
Clostridium	0	0	0	0	0	0	0.024043 8	0.010437 9	0
Flavobacterium	0.410155 6	0.002725 6	0	0	0	0	0.002003 6	0.009419 6	0
Mediterraneibacter	0	0	0	0	0	0.026664	0	0.007382 9	0
Gemmiger	0.092878 2	0.021350 2	0	0.041917 7	0.016121 8	0.022931	0.012594 4	0.005600 8	0
Phocaeicola	0.288803 8	0.022713	0.008009 2	0.030390 4	0.108075 8	0.002666 4	0.003721 1	0.005091 6	0
Escherichia	0.013558 9	0	0	0	0	0	0	0.002800 4	0
Zoogloea	0	0	0	0	0	0	0	0.002545 8	0
Stenotrophomonas	0.033897 2	0	0	0	0	0	0	0.002291 2	0
Faecousia	0	0	0	0	0	0	0.002003 6	0.001782 1	0

Terrisporobacter	0	0	0	0	0	0	0.003434 8	0.001527 5	0
Limosilactobacillus	0.022372 1	0.075861 5	0.038632 4	0.011003 4	0.077623 5	0.054394 5	0	0.001527 5	0
Fimimorpha	0	0	0	0	0	0	0	0.001527 5	0
Eubacterium	0.008813 3	0	0	0	0	0	0.002003 6	0.001272 9	0
Anaerofilum	0.001355 9	0	0	0	0	0	0	0.001272 9	0
Caproicibacterium	0	0	0	0	0	0	0	0.001272 9	0
Lawsonibacter	0	0	0	0	0	0	0	0.001272 9	0
Anaerotruncus	0	0	0	0	0	0	0	0.001018 3	0
Sharpea	0	0	0	0	0	0	0.222118 5	0	0
Sodaliphilus	0.004067 7	0	0	0	0	0	0.046370 1	0	0
Phytobacter	0.09559	0.484241 7	0.447570 6	0.371495 9	0.398268 4	0.164783 3	0.040359 2	0	0
Bulleidia	0	0	0	0	0	0	0.038641 8	0	0
Ruminococcus	0.010169 1	0.007268 2	0	0	0	0	0.031485 9	0	0
Holdemanella	0.089488 5	0.002271 3	0	0.006287 7	0.005971	0.089057 6	0.026047 4	0	0

Citrobacter	0	0.215319 5	0.153116 3	0.156143 6	0.173160 2	0.138652 6	0.025474 9	0	0
Intestinibacter	0	0	0	0	0	0	0.013739 3	0	0
Intestinimonas	0	0	0	0	0	0	0.012308 1	0	0
Prevotella	0.012203	0.024984 3	0	0.026722 6	0	0	0.011735 6	0	0
Parafannyhessea	0	0	0	0	0	0	0.011735 6	0	0
Absicoccus	0	0	0	0	0	0	0.009732	0	0
Alloprevotella	0.002711 8	0	0	0	0	0	0.009445 8	0	0
Blautia	0.014236 8	0	0	0.003667 8	0.006568 1	0.007999 2	0.008300 8	0	0
Copromorpha	0	0	0	0	0	0	0.006010 9	0	0
Ruthenibacterium	0	0	0	0	0	0	0.005438 5	0	0
Cupriavidus	0	0	0.000942 3	0	0	0.001066 6	0.005152 2	0	0
Sphingomicrobium	0.098301 8	0	0	0.004715 7	0	0	0.004007 3	0	0
Lachnospira	0	0.001817	0	0	0	0	0.003721 1	0	0
Paratractidigestivibacter	0	0	0	0	0	0	0.003434 8	0	0
Parabacteroides	0	0.000908	0	0	0	0	0.002862	0	0

		5					4		
							0.002576		
Senegalimassilia	0	0	0	0	0	0	1	0	0
							0.002289		
Caproicibacter	0	0	0	0	0	0	9	0	0
							0.002289		
Emergencia	0	0	0	0	0	0	9	0	0
	0.003389	0.002725		0.007859	0.004179		0.002003		
Collinsella	7	6	0	6	7	0	6	0	0
							0.002003		
Hyphomicrobium	0	0	0	0	0	0	6	0	0
							0.001717		
Dorea	0	0	0	0	0	0	4	0	0
		0.000908					0.001431		
Microvirga	0	5	0	0	0.057322	0	2	0	0
							0.001431		
Fimenecus	0	0	0	0	0	0	2	0	0
							0.001144		
Planktophila	0	0	0	0	0	0	9	0	0
							0.001144		
Reyranella	0	0	0	0	0	0	9	0	0
					0.001791		0.000858		
Cryptobacteroides	0	0	0	0	3	0	7	0	0
				0.003667			0.000858		
Phascolarctobacterium	0	0	0	8	0	0	7	0	0
	0.002711						0.000858		
Butyricicoccus	8	0	0	0	0	0	7	0	0
Merdicola	0	0	0	0	0	0	0.000858	0	0

Tumebacillus	0	0	0	0	0	0	7 0.000858 7	0	0
Onthovivens	0	0	0	0	0	0	0.000572 5	0	0
Asaia	0.023050 1	0.014536 3	0.016489 4	0	0	0.051194 8	0	0	0
Oryzihumus	0	0	0	0	0	0.034663 2	0	0	0
Salinicoccus	0	0	0	0	0	0.028797 1	0	0	0
Ligilactobacillus	0.006101 5	0.046788 8	0.029209 9	0.017291 1	0.020301 5	0.020264 6	0	0	0
Streptococcus	0.019660 4	0	0	0.005239 7	0	0.011732 1	0	0	0
Massilia	0.010169 1	0	0	0.005239 7	0	0.009065 7	0	0	0
Cutibacterium	0	0	0	0	0	0.006399 4	0	0	0
Lachnoclostridium	0	0	0	0	0	0.006399 4	0	0	0
Mycobacterium	0.020338 3	0	0	0	0	0.003733	0	0	0
Spirillospora	0	0	0	0	0	0.003199 7	0	0	0
Bifidobacterium	0.021016 2	0.002271 3	0	0.009431 5	0	0.002666 4	0	0	0
Moraxella	0	0.003179	0	0	0	0.002666	0	0	0

		8				4			
						0.002666			
Ktedonobacter	0	0	0	0	0	4	0	0	0
			0.001884		0.008359	0.002133			
Sulfurimonas	0	0	5	0	5	1	0	0	0
						0.002133			
Kitasatospora	0	0	0	0	0	1	0	0	0
						0.002133			
Noviherbaspirillum	0	0	0	0	0	1	0	0	0
						0.002133			
Paludibaculum	0	0	0	0	0	1	0	0	0
				0.001571		0.001599			
Solirubrobacter	0	0	0	9	0	8	0	0	0
						0.001599			
Dormibacter	0	0	0	0	0	8	0	0	0
						0.001066			
Rubrimentiphilum	0	0	0	0	0	6	0	0	0
					0.057919				
Yaniella	0	0	0	0	1	0	0	0	0
					0.043588				
Blastococcus	0	0	0	0	6	0	0	0	0
	0.002711				0.008956				
Alistipes	8	0	0	0	6	0	0	0	0
Niallia	0	0	0	0	0.005971	0	0	0	0
					0.002985				
Erysipelatoclostridium	0	0	0	0	5	0	0	0	0
					0.002985				
Rubneribacter	0	0	0	0	5	0	0	0	0

Acidithrix	0	0	0	0	0.002388 4	0	0	0	0
Sulfotelmatobacter	0	0	0	0	0.001791 3	0	0	0	0
Trebonia	0	0	0	0	0.001791 3	0	0	0	0
Udaeobacter	0	0	0	0	0.001194 2	0	0	0	0
Fusobacterium	0	0.013627 8	0	0.008383 5	0	0	0	0	0
Porphyromonas	0.020338 3	0.005451 1	0	0.005763 7	0	0	0	0	0
Bergeyella	0.006779 4	0.003634 1	0	0.005239 7	0	0	0	0	0
Barnesiella	0	0	0	0.004715 7	0	0	0	0	0
Jatrophihabitans	0.292871 4	0	0	0.003667 8	0	0	0	0	0
Adlercreutzia	0.006779 4	0.008176 7	0	0.003143 8	0	0	0	0	0
Aureimonas	0.002711 8	0	0	0.002619 9	0	0	0	0	0
Nocardioides	0.050845 7	0	0	0.002095 9	0	0	0	0	0
Slackia	0	0	0	0.002095 9	0	0	0	0	0
Gordonibacter	0	0	0	0.001571 9	0	0	0	0	0

Amycolatopsis	0	0	0	0.001047 9	0	0	0	0	0
Christensenella	0	0	0	0.001047 9	0	0	0	0	0
Metallibacterium	0	0	0.001884 5	0	0	0	0	0	0
Humisphaera	0.007457 4	0	0.001413 4	0	0	0	0	0	0
Acidulodesulfobacterium	0	0	0.000942 3	0	0	0	0	0	0
Patulibacter	0	0	0.000942 3	0	0	0	0	0	0
Paracoccus	0	0.007268 2	0	0	0	0	0	0	0
Trichococcus	0	0.006813 9	0	0	0	0	0	0	0
Romboutsia	0	0.005905 4	0	0	0	0	0	0	0
Frederiksenia	0	0.002271 3	0	0	0	0	0	0	0
PMG-095	0	0.002271 3	0	0	0	0	0	0	0
Scatomonas	0	0.002271 3	0	0	0	0	0	0	0
Desulfovibrio	0.002033 8	0.001817	0	0	0	0	0	0	0
Oceanivirga	0.002033 8	0.001817	0	0	0	0	0	0	0

Methyloceanibacter	0.0013559	0.0013628	0	0	0	0	0	0	0
Desulfofustis	0	0.0013628	0	0	0	0	0	0	0
Oribacterium	0	0.0013628	0	0	0	0	0	0	0
Phycicoccus	0	0.0013628	0	0	0	0	0	0	0
Terriglobus	0.0366089	0.0009085	0	0	0	0	0	0	0
Bruticola	0.0020338	0.0009085	0	0	0	0	0	0	0
Humimicrobium	0	0.0009085	0	0	0	0	0	0	0
Veillonella	0	0.0009085	0	0	0	0	0	0	0
Frondihabitans	0.2949053	0	0	0	0	0	0	0	0
Pigmentiphaga	0.2928714	0	0	0	0	0	0	0	0
Duganella	0.2454154	0	0	0	0	0	0	0	0
Paraburkholderia	0.2271109	0	0	0	0	0	0	0	0
Frankia	0.2196536	0	0	0	0	0	0	0	0
Caballeronia	0.1837226	0	0	0	0	0	0	0	0

Tardiphaga	0.170163 7	0	0	0	0	0	0	0	0
Pararhizobium	0.150503 4	0	0	0	0	0	0	0	0
Rhizorhabdus	0.138300 4	0	0	0	0	0	0	0	0
Kineococcus	0.105081 2	0	0	0	0	0	0	0	0
Aquisphaera	0.101691 5	0	0	0	0	0	0	0	0
Roseomonas	0.09559	0	0	0	0	0	0	0	0
Bosea	0.094234 1	0	0	0	0	0	0	0	0
Variovorax	0.092200 3	0	0	0	0	0	0	0	0
Fibrobacter	0.088132 6	0	0	0	0	0	0	0	0
Janthinobacterium	0.084742 9	0	0	0	0	0	0	0	0
Spirosoma	0.079997 3	0	0	0	0	0	0	0	0
Subtercola	0.075251 7	0	0	0	0	0	0	0	0
Beijerinckia	0.073217 9	0	0	0	0	0	0	0	0
Mucilaginibacter	0.073217 9	0	0	0	0	0	0	0	0
Brevundimonas	0.072539	0	0	0	0	0	0	0	0

	9								
	0.068472								
Yonghaparkia	3	0	0	0	0	0	0	0	0
	0.066438								
Rubrivivax	4	0	0	0	0	0	0	0	0
	0.051523								
Azospirillum	7	0	0	0	0	0	0	0	0
	0.039320								
Fimbriimonas	7	0	0	0	0	0	0	0	0
	0.031863								
Finegoldia	3	0	0	0	0	0	0	0	0
	0.031185								
Rhizomicrobium	4	0	0	0	0	0	0	0	0
	0.020338								
Faecalibacillus	3	0	0	0	0	0	0	0	0
	0.019660								
Chryseobacterium	4	0	0	0	0	0	0	0	0
	0.018982								
Clavibacter	4	0	0	0	0	0	0	0	0
	0.014914								
Williamsia	7	0	0	0	0	0	0	0	0
	0.014236								
Aurantimonas	8	0	0	0	0	0	0	0	0
	0.014236								
Sphingobium	8	0	0	0	0	0	0	0	0
Granulicella	0.012203	0	0	0	0	0	0	0	0
Onthenecus	0.012203	0	0	0	0	0	0	0	0
Hymenobacter	0.010847	0	0	0	0	0	0	0	0

	1 0.010169								
Mesorhizobium	1 0.008813	0	0	0	0	0	0	0	0
Acidisoma	3 0.008813	0	0	0	0	0	0	0	0
Rhodococcus	3 0.008135	0	0	0	0	0	0	0	0
Rhizobacter	3 0.006779	0	0	0	0	0	0	0	0
Hansschlegelia	4 0.006101	0	0	0	0	0	0	0	0
Granulicatella	5 0.005423	0	0	0	0	0	0	0	0
Allobaculum	5 0.005423	0	0	0	0	0	0	0	0
Alsobacter	5 0.005423	0	0	0	0	0	0	0	0
Luteimonas	5 0.005423	0	0	0	0	0	0	0	0
Paraclostridium	5 0.004745	0	0	0	0	0	0	0	0
Aeromicrobium	6 0.004745	0	0	0	0	0	0	0	0
Kineobactrum	6 0.004745	0	0	0	0	0	0	0	0
Limivicinus	6	0	0	0	0	0	0	0	0
Megasphaera	0.004745	0	0	0	0	0	0	0	0

	6								
	0.004745								
Micrococcus	6	0	0	0	0	0	0	0	0
	0.003389								
Salipiger	7	0	0	0	0	0	0	0	0
	0.003389								
Sphingobacterium	7	0	0	0	0	0	0	0	0
	0.002711								
Helcococcus	8	0	0	0	0	0	0	0	0
	0.002711								
Kaistia	8	0	0	0	0	0	0	0	0
	0.002033								
Oceanimonas	8	0	0	0	0	0	0	0	0
	0.002033								
Perlucidibaca	8	0	0	0	0	0	0	0	0
	0.001355								
Arcanobacterium	9	0	0	0	0	0	0	0	0
Fungi									
	18.77323	96.47373	97.76563	98.31334	89.84802	64.76185	24.39878	2.823772	77.69515
Saccharomyces	4	5	9	6	6	2	9	9	1
	10.54832	0.009980	0.025274	0.005405	0.234811		16.57032	67.31224	2.603075
Pichia	7	9	7	9	4	17.52541	1	1	8
	2.416356	0.003659		0.001138				0.994234	
Candida	9	7	0	1	0	0	0	2	0
	14.68401	0.032604	0.020382	0.007113		0.029907			
Yarrowia	5	4	8	1	0.005135	6	0	0	0.016013
	9.526022	0.015304				0.009505			
Kluyveromyces	3	1	0	0	0	5	0	0	0

Hanseniaspora	0	3.213861 5	1.990990 8	0.928115 2	0.710269 4	3.933897 2	3.427740 8	2.757244 2	0.628991 2
Leotiomycetes	15.52044 6	0.012309 8	0	0.000853 6	0	0.018779 2	0	0	0
Alternaria	12.22119	0.043250 7	0.006522 5	0.003414 3	0.001400 5	0	0	0	0
Brettanomyces	0	0	0.023236 4	0.645014 4	9.126833 2	13.50967 2	55.57456 1	22.16513 9	18.96773 7
Cladosporium	3.763940 5	0.003992 4	0.004076 6	0.002560 7	0	0	0	0.443524 5	0
Sistotrema	2.416356 9	0.010646 3	0.001630 6	0.001707 1	0	0	0	0	0
Helotiales	2.276951 7	0	0	0	0	0	0	0	0
Boeremia	1.765799 3	0.005988 6	0	0	0	0	0	0	0
Sympodiomycopsis	1.16171	0	0	0	0	0	0	0	0
Aspergillus	1.115241 6	0.005988 6	0	0	0	0	0.022289 5	0	0
Bullera	1.022304 8	0	0.004076 6	0	0	0	0	0	0
Vishniacozyma	0.882899 6	0	0	0	0	0	0	0	0
Clavispora	0.697026	0.005988 6	0	0.001138 1	0	0	0	0	0
Papiliotrema	0.650557 6	0.007984 7	0	0.001422 6	0	0	0	0	0
Ophiosphaerella	0.557620	0	0	0	0	0	0	0.543317	0

	8							6	
								0.358515	
Agaricomycetes	0	0	0	0	0	0	0	7	0
								0.247634	
Debaryomyces	0	0	0	0	0	0	0	5	0
								1.644736	
Exobasidium	0	0	0	0	0	0	0	8	0
		0.121434	0.151647	0.083934	0.049249	0.210976	0.006299		
Kurtzmaniella	0	7	9	4	7	3	2	0	0.016013
			0.002038						
Malassezia	0	0	3	0	0	0	0	0	0
					0.024274			0.709639	
Malassezia	0	0	0	0	7	0	0	3	0
				0.001422					0.018575
Penicillium	0	0	0	6	0	0	0	0	1
		0.002994							
Rhodotorula	0	3	0	0	0	0	0	0	0
		0.010313							
Sporobolomyces	0	6	0	0	0	0	0	0	0
		0.001996	0.004484	0.001991					0.054444
Starmerella	0	2	2	7	0	0	0	0	3
		0.003659		0.001422					
Tetracladium	0	7	0	6	0	0	0	0	0
Yamadazyma	0	0.014306	0	0	0	0	0	0	0