UNIVERSIDAD DE CÓRDOBA

Tesis Doctoral

Programa de Doctorado: Biociencias y Ciencias Agroalimentarias

Título de la Tesis: "Mejora de la elaboración de vinagre a través del estudio integrado del microbioma y metaboloma"

Thesis title: "Improvement of vinegar processing through integrated study of the microbiome and metabolome"

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TITULO: *Improvement of vinegar processing through integrated study of the microbiome and metabolome*

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TÍTULO DE LA TESIS:
MEJORA DE LA ELABORACIÓN DE VINAGRE A TRAVÉS DEL ESTUDIO INTEGRADO DEL MICROBIOMA Y METABOLOMA

THESIS TITLE:
IMPROVEMENT OF VINEGAR PROCESSING THROUGH INTEGRATED STUDY OF THE MICROBIOME AND METABOLOME

DOCTORANDO: JUAN JESÚS ROMÁN CAMACHO

INFORME RAZONADO DE LOS DIRECTORES DE LA TESIS

Los doctores **JUAN CARLOS GARCÍA MAURICIO**, Catedrático de Universidad del Área de Microbiología del Departamento de Química Agrícola, Edafología y Microbiología de la Universidad de Córdoba e **ISIDORO GARCÍA GARCÍA**, Catedrático de Universidad del Área de Ingeniería Química del Departamento de Química Inorgánica e Ingeniería Química de la Universidad de Córdoba.

INFORMAN:

Que el trabajo de investigación presentado por Don **Juan Jesús Román Camacho**, titulado "MEJORA DE LA ELABORACIÓN DE VINAGRE A TRAVÉS DEL ESTUDIO INTEGRADO DEL MICROBIOMA Y METABOLOMA" se ha realizado bajo la dirección y supervisión de ambos directores en los laboratorios de las Áreas de Microbiología e Ingeniería Química de la Universidad de Córdoba y en el Departamento de Microbiología (Freising, Alemania) bajo la tutela del Prof. Wolfgang Liebl de la Universidad Técnica de Múnich (Alemania) y reúne las condiciones exigidas para su presentación y defensa pública como Tesis Doctoral con mención internacional y en la modalidad de compendio de publicaciones.

El trabajo presentado por D. **Juan Jesús Román Camacho**, Graduado en Biología por la Universidad de Córdoba, se encuadra en las líneas de investigación "Enología y Biotecnología de las Fermentaciones" y "Análisis de microbiotas complejas mediante técnicas ómicas y estudio de aspectos moleculares de bioprocesos" de reciente implantación en los grupos de investigación AGR-146 (VITICULTURA Y ENOLOGÍA, "Vitenol", http://www.uco.es/investiga/grupos/vitenol/), del que es miembro, y RNM-940 (BIOPROCESOS E INGENIERÍA DE PRODUCTOS, "BioPrEn", https://www.uco.es/BioPrEn/). Estos grupos están formados por un equipo de investigación multidisciplinar compuesto por químicos, microbiólogos, enólogos, ingenieros químicos, de ambos departamentos citados anteriormente, que trabajan de una manera colaborativa que favorece una sinergia, que por separado no se podría conseguir. Esta línea de investigación tiene como objetivo principal estudiar procesos

fermentativos, como son la fermentaciones alcohólica y acética, usando herramientas ómicas para comprender mejor los bioprocesos y como objetivo final mejorar la calidad de los productos clásicos, como vinos y vinagres, y elaborar nuevos con gran interés industrial, económico y social.

El doctorando **Juan Jesús Román Camacho** ha desarrollado, desde su incorporación al programa de doctorado Biociencias y Ciencias Agroalimentarias, una intensa y excelente actividad investigadora y docente, cuya novedad e interés para la comunidad científica internacional está avalada por las publicaciones incorporadas en la Tesis Doctoral como primer autor de cuatro artículos de investigación en revistas internacionales de un elevado índice de impacto indexada en el primer cuartil del Journal Citation Reports (JCR) en las categorías de Microbiología y Ciencia y Tecnología de los Alimentos.

El doctorando ha realizado una extensa revisión bibliográfica de las publicaciones más relevantes y actuales sobre su tema de investigación, que le ha permitido elaborar una importante introducción de la Tesis Doctoral. Básicamente, esta introducción consiste en una descripción de la situación actual del proceso de elaboración del vinagre, centrándose fundamentalmente en la microbiota responsable del proceso. Sin duda, esta exhaustiva y actualizada introducción servirá como base para la publicación de una o dos revisiones sobre el tema.

La originalidad de dicha Tesis Doctoral estriba en el uso de herramientas ómicas como la metagenómica, metaproteómica y metabolómica de una manera integrada para el estudio de la elaboración de vinagre en cultivo sumergido.

Las investigaciones realizadas en la presente Tesis se han dirigido, fundamentalmente, a dilucidar la microbiota presente durante la fermentación acética en varios sustratos alcohólicos (etanol vínico, vino fino, y cerveza artesana) y cómo funciona en las condiciones extremas de elaboración de vinagre.

Los objetivos planteados tienen una sólida coherencia y han facilitado la obtención de unos resultados importantes e innovadores dentro del área de Ciencias y Tecnología de los Alimentos. La colaboración interdisciplinar Microbiología – Ingeniería Química en el ámbito de la elaboración de vinagre ha hecho posible la consecución de los objetivos propuestos en la Tesis, de manera que principalmente cada uno forma un capítulo de esta memoria y, a su vez, cada capítulo se corresponde con cada artículo publicado.

El conocimiento generado en esta Tesis Doctoral y su transferencia al sector permitirá un mejor control de calidad del proceso de elaboración de vinagres, una diversificación de los productos elaborados y, por tanto, un mayor grado de innovación en esta industria agroalimentaria tan importante en Andalucía.

El doctorando ha realizado una estancia de tres meses de duración en la Universidad Técnica de Múnich (Alemania), financiada por el IDEP de la Universidad de Córdoba, que ha sido tutorizada por el Dr. Wolfgang Liebl. Durante esta estancia ha contribuido a mejorar sus conocimientos sobre metagenómica del vinagre y a ampliar las aplicaciones de esta metodología a otros bioprocesos a partir de sustratos complejos. Además, esta estancia le permite solicitar la mención de Doctorado Internacional.

Por todo lo expuesto, se considera que la investigación desarrollada y recogida en la presente memoria reúne los requisitos de interés, originalidad, novedad y calidad científica exigidos para una Tesis Doctoral por la Universidad de Córdoba, con mención de Doctorado Internacional y por Compendio de Artículos, y se emite este informe favorable para la presentación de la Tesis Doctoral de Juan Jesús Román Camacho.

Este trabajo se ha realizado gracias a los siguientes proyectos:

La investigación ha sido realizada con cofinanciación de la Consejería de Economía, Conocimiento, Empresas y Universidad en el ámbito del Plan Andaluz de Investigación, Desarrollo e Innovación (PAIDI 2020) y FEDER. Ref. PY20_00590. También, con la cofinanciación del MICINN-Plan Estatal de Investigación Científica, Técnica y de Innovación 2021-2023 ayudas a «Proyectos de Generación de Conocimiento» y FEDER. Ref. PID2021-127766OB-I00.

Córdoba, 8 de noviembre de 2022

Firma de los directores

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REPORT OF PUBLICATIONS ACCORDING TO THE JOURNAL CITATION REPORT (JCR)

This Doctoral Thesis is presented by "Compendio de Publicaciones" and therefore, the following is a report including these publications, as well as those not derived from the Doctoral Thesis. Information about their respective journals, whose impact factors can be found in the *Journal Citation Report* (*JCR*) of *Web of Science* (*WOS*), is also shown:

Publication No. 1
Authors: Román-Camacho, J. J., Santos-Dueñas, I. M., García-García, I., Moreno-García, J., García-Martínez, T., Mauricio, J. C. **Title**: Metaproteomics of microbiota involved in submerged culture production of alcohol wine vinegar: A first approach **Journal:** International Journal of Food Microbiology **Category of the Journal:** Food Science and Technology **Year of publication:** 2020 **Impact factor (2020):** 5.277 **Quartile:** Q1 (25/143) **DOI:** 10.1016/j.ijfoodmicro.2020.108797
Publication No. 2
Authors: Román-Camacho, J. J., Mauricio, J. C., Santos-Dueñas, I. M., García-Martínez, T., García-García, I. **Title**: Functional metaproteomic analysis of alcohol vinegar microbiota during an acetification process: A quantitative proteomic approach **Journal:** Food Microbiology **Category of the Journal:** Food Science and Technology **Year of publication:** 2021 **Impact factor (2021):** 6.374 **Quartile:** Q1 (22/143) **DOI:** 10.1016/j.fm.2021.103799

Publication No. 3

Authors: Román-Camacho, J. J., Mauricio, J. C., Santos-Dueñas, I. M., García-Martínez, T., García-García, I.

Title: Unraveling the role of acetic acid bacteria comparing two acetification profiles from natural raw materials: a quantitative approach in *Komagataeibacter europaeus*

Journal: Frontiers in Microbiology

Category of the Journal: Microbiology

Year of publication: 2022

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Quartile: Q1 (34/136)

DOI: 10.3389/fmicb.2022.840119

Publication No. 4 (accepted)

Authors: Román-Camacho, J. J., García-García, I., Santos-Dueñas, I. M., Ehrenreich, A., Liebl, W., García-Martínez, T., Mauricio, J. C.

Title: Combining omics tools for the characterization of the microbiota of diverse vinegars obtained by submerged culture: 16S rRNA Amplicon Sequencing and MALDI-TOF MS

Journal: Frontiers in Microbiology

Category of the Journal: Microbiology

Year of publication: 2022

Impact factor (2021): 6.064

Quartile: Q1 (34/136)

DOI:

Publication No. 5 (not derived from the Doctoral Thesis)

Authors: Porras-Agüera, J. A., Román-Camacho, J. J., Moreno-García, J., Mauricio, J. C., Moreno, J., García-Martínez, T.

Title: Effect of endogenous CO_2 overpressure on the yeast "stressome" during the "prise de mousse" of sparkling wine

Journal: Food Microbiology

Category of the Journal: Food Science and Technology

Year of publication: 2020

Impact factor (2020): 5.516

Quartile: Q1 (21/143)

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La realización de una Tesis Doctoral se podría asemejar a subirte en una montaña rusa, en la cual, hay constantes subidas, que traen momentos de alegría y satisfacción al igual que bajadas, en las que abundan sensaciones como la tristeza, la desesperación y el estrés. Llegar a la meta con éxito no solo requiere de un enorme trabajo, sacrificio y constancia por parte del doctorando, sino también de contar con un entorno, tanto profesional como personal, idóneo. Este ha sido mi caso, es por ello por lo que, a continuación, me gustaría agradecer a todas aquellas personas, instituciones y empresas que han contribuido, de un modo u otro, a la realización de este trabajo.

En primer lugar, me gustaría agradecer a mis directores de Tesis, los Profesores Juan Carlos García Mauricio e Isidoro García García, por brindarme la oportunidad de realizar esta Tesis Doctoral. Al Dr. Juan Carlos, por abrirme las puertas del área de Microbiología cuando era estudiante. Por confiar en mí desde el primer momento, darme la oportunidad de comenzar en el mundo de la investigación y haberme formado. Nunca tendré suficientes palabras de agradecimiento y aunque finalice esta Tesis Doctoral, siempre serás, profesionalmente, un padre y un referente para mí. Al Dr. Isidoro, por abrirme las puertas del área de Ingeniería Química y haberme formado en una rama que desconocía pero que ahora me fascina. Por tu infinita dedicación, paciencia y por tener siempre un consejo lleno de sabiduría o una palabra amable en el momento oportuno. Por potenciar mi entusiasmo por la investigación y haberme enseñado a disfrutar del camino. No cabe duda de que vuestra enorme dedicación, esfuerzo y apoyo incondicional a lo largo de estos años me han permitido, no sólo terminar este trabajo con una enorme satisfacción, sino ser hoy mejor profesional y mejor persona.

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A Irene, mi compañera, la persona que más me ha ayudado a sacar esta Tesis Doctoral. Gracias por aguantar todos mis momentos de agobio y mal humor con paciencia, por

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Esta Tesis Doctoral está dedicada a todos vosotros, ¡¡MUCHAS GRACIAS!!

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ABBREVIATIONS AND ACRONYMS

2D-DIGE	Two-Dimensional-Differential Gel Electrophoresis
2DE	Two-Dimensional Electrophoresis
AAB	Acetic Acid Bacteria
ABC	ATP-Binding Cassette
AFLP	Amplified Fragment Length Polymorphism
ANOVA	Analysis of Variance
ASV	Amplicon Sequence Variant
ATP	Adenosine Triphosphate
AW	Synthetic alcohol-based medium
AW.EL	At the End of Loading-Alcohol medium acetification
AW.S	Substrate-Alcohol medium
AW.UL	Just before Unloading-Alcohol medium acetification
B	Craft Beer
B.EL	At the End of Loading-Craft Beer acetification
B.S	Substrate-Craft Beer
B.UL	Just before Unloading-Craft Beer acetification
BAA	Bacterias del Ácido Acético
BCAA	Branched-Chain Amino Acids
BLAST	Basic Local Alignment Search Tool
CE-MS	Capillary Electrophoresis-Mass Spectrometry
CPS	Capsular Polysaccharides
DGGE	Denaturing Gradient Gel Electrophoresis
DHPLC	Denaturing High-Performance Liquid Chromatography
DIGE	Differential Gel Electrophoresis
DNA	Deoxyribonucleic Acid
EDP	Entner Doudoroff Pathway
ERIC	Enterobacterial Repetitive Intergenic Consensus
FW	Fine wine
FW.EL	At the End of Loading-Fine Wine acetification
FW.S	Substrate-Fine Wine
FW.UL	Just before Unloading-Fine Wine acetification
G + C	Guanine + Cytosine
GC-MS	Gas Chromatography-Mass Spectrometry
GO	Gen Ontology
HPLC-MS	High-Performance Liquid Chromatography-Mass Spectrometry
IEF	Isoelectric Focusing
iTRAQ	Isobaric Tags for Relative and Absolute Quantitation
ITS	Intergenic Spacer Region
KEGG	Kyoto Encyclopedia of Genes and Genomes
LAB	Lactic Acid Bacteria
LC	Liquid Chromatography
LC-MS	Liquid Chromatography-Mass Spectrometry

LC-MS/MS	Liquid Chromatography with Tandem Mass Spectrometry
LPSN	List of Prokaryotic names with Standing in Nomenclature
MALDI-TOF MS	Matrix-Assisted Laser Desorption Ionization-Time Of Flight Mass Spectrometry
MANOVA	Multivariate Analysis of Variance
MLST	Multi-Locus Sequence Typing
MS	Mass Spectrometry
NGS	Next-Generation Sequencing
NMR	Nuclear Magnetic Resonance
OMP	Outer Membrane Protein
OTU	Operational Taxonomic Unit
PC	Phosphatidylcholine
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
PDMS	Polydimethylsiloxane
PDO	Protected Designation of Origin
PPP	Pentose Phosphate Pathway
QIIME2	Quantitative Insights Into Microbial Ecology
qPCR	Quantitative Real-Time Polymerase Chain Reaction
R	Rough cell surface
RAPD	Random Amplified Polymorphic DNA
REP	Repetitive Extragenic Palindromic
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
rRNA	Ribosomal Ribonucleic Acid
S	Smooth cell surface
SBSE	Stir Bar Sorptive Extraction
SCADA	Supervisory Control and Data Acquisition
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SEM	Scanning Electron Microscopy
SSF	Solid-State Fermentation
STRING	Search Tool for the Retrieval of Interacting Genes/Proteins
TCA	Tricarboxylic Acid Cycle
TDS	Thermodesorption System
THBH	Tetrahydroxybacteriohopane
tRNA	Transfer Ribonucleic Acid
VBNC	Viable But Non-Culturable

RESUMEN

Introducción y motivación de la Tesis

La elaboración de vinagre de forma industrial se realiza a partir de un medio de origen alcohólico en el que interviene un cultivo mixto de bacterias acéticas (BAA) para llevar a cabo un proceso de biotransformación del etanol en ácido acético. A pesar de que el proceso de acetificación es bien conocido desde un punto de vista práctico y técnico, existen todavía numerosos aspectos fundamentales que no se han estudiado de forma exhaustiva, especialmente, aquellos que controlan, en último término, la actividad y el comportamiento de las complejas microbiotas responsables del proceso. Se sabe que estas comunidades microbianas mayormente están conformadas por unas pocas especies de BAA, aerobias estrictas y principales responsables del mencionado proceso (Gullo et al., 2014; Peters et al., 2017). No obstante, fracciones menores tanto de BAA como de otros microorganismos pueden coexistir en el medio con las especies predominantes y contribuir a la función de la comunidad (Trček et al., 2016; Peng et al., 2021). Los miembros de estas microbiotas y, especialmente, las BAA, debido a sus condiciones de crecimiento tan particulares basadas principalmente en la necesidad de un medio líquido rico en etanol como fuente de carbono, homogéneo y con constante aporte de oxígeno disuelto, son difíciles de aislar y cultivar fuera de estos entornos dónde normalmente realizan su actividad (Mamlouk and Gullo, 2013; Gullo et al., 2014). En este contexto, la identificación, así como los estudios de biodiversidad y comportamiento de estos microorganismos a nivel molecular plantean numerosas dificultades. Estos aspectos no sólo implican un desafío para mejorar la comprensión de conceptos científicos-básicos de estas bacterias, sino que dificultan el control de la calidad final de los productos obtenidos, ya que depende de la composición microbiana, la materia prima y las condiciones operativas (Mas et al., 2014).

En el contexto que se plantea, las ciencias ómicas ofrecen en la actualidad múltiples posibilidades para la identificación y caracterización de comunidades microbianas en su medio natural, sin necesidad de aislamiento. En el campo de la producción de vinagre, los estudios existentes están mayormente centrados en metagenómica y/o metabolómica de vinagres tradicionales, tanto aquellos obtenidos mediante fermentación en estado sólido como en superficie, así como estudios moleculares de especies concretas (Xia et al., 2016; Peters et al., 2017; Wu et al., 2017; Zhu et al., 2018; Wang et al., 2021). Este

trabajo, enfocado en el uso del cultivo sumergido como método de producción y numerosas herramientas ómicas, pretende contribuir a ampliar el conocimiento actual que existe en esta área de investigación.

Contenido de la investigación

En la presente Tesis Doctoral, se caracterizó y comparó el desarrollo de tres perfiles de acetificación para estudiar la evolución de las variables del sistema y el efecto de las tres materias primas empleadas: un medio sintético de alcohol, un vino fino y una cerveza artesana, sobre la composición y la actividad de la microbiota responsable del proceso. Con este objetivo, se emplearon diferentes herramientas ómicas basadas en metaproteómica (LC-MS/MS), metagenómica (secuenciación del ARNr 16S), huella de proteínas (MALDI-TOF MS) y metabolómica (SBSE acoplada con GC-MS), así como el cultivo sumergido, trabajando en modo semicontinuo, para la producción de vinagre a escala de laboratorio, tratando de imitar los procedimientos industriales.

En primer lugar, la caracterización de los perfiles de acetificación de las materias primas de trabajo, desde un enfoque metaproteómico, permitió describir la composición y el comportamiento de la microbiota responsable del proceso. El género *Komagataeibacter*, representado principalmente por la especie *Komagataeibacter europaeus* y seguida de otras especies relacionadas, conformaron la microbiota predominante. Adicionalmente, este análisis reveló una fracción menor de microorganismos compuesta por géneros típicos de la familia Acetobacteraceae, nunca descritos en vinagre hasta la fecha. Posteriores estudios de metagenómica y huella proteica confirmaron la predominancia del género *Komagataeibacter* y permitió describir nuevos géneros bacterianos en estos medios e incluso grupos de arqueas. A partir de los estudios de metaproteómica, análisis cuantitativos centrados en el perfil de proteínas de la especie mayoritaria, *K. europaeus*, permitieron diferenciar entre los principales procesos biológicos que tienen lugar a lo largo del ciclo de acetificación en base a las variaciones en la actividad de las proteínas asociadas. Los resultados procedentes de la caracterización y comparación de dos perfiles de acetificación de materias primas naturales: vino fino y cerveza artesana, permitieron sugerir una estrategia molecular en la que *K. europaeus* puede asegurar su supervivencia a través del uso de nutrientes presentes en cada sustrato. En base a ello, la metabolización del ácido acético, procedente de la oxidación incompleta del etanol, mediante el ciclo de Krebs y otras vías metabólicas relacionadas (ruta de las pentosas

fosfato y glucolisis), suministrando precursores biosintéticos (aminoácidos y ácidos nucleicos), así como mecanismos de membrana para liberar el ácido acético, pueden ser procesos de interés biotecnológico para la producción de vinagre mediante cultivo sumergido. Por último, un estudio preliminar a nivel metabolómico condujo a la caracterización del "volatiloma" y la diferenciación de volátiles minoritarios clave tanto de las materias primas como de las respectivas acetificaciones, permitiendo establecer diferencias significativas, siendo éstas más evidentes entre el medio sintético y los medios naturales.

Conclusión

La investigación realizada en este trabajo ha permitido caracterizar y confirmar la composición de la microbiota presente durante tres perfiles de acetificación, con *K. europaeus* como especie predominante seguida de una fracción menos abundante de microorganismos como especies estrechamente relacionadas, especies de otros géneros típicos de BAA, grupos bacterianos diferentes a las BAA e incluso arqueas. Desde un punto de vista cuantitativo, se ha demostrado que el uso de diversas materias primas no influye directamente en la composición microbiana, mayormente común, pero sí en la actividad, comportamiento y estrategias moleculares empleadas durante la acetificación.

Estos hallazgos pueden contribuir a mejorar el conocimiento actual que existe acerca de la composición y el papel de las comunidades microbianas responsables del proceso de acetificación, así como ampliar el uso de nuevas materias primas para la elaboración de vinagre mediante cultivo sumergido. A su vez, estos logros podrían conducir a la mejora de las condiciones de operación para la obtención de nuevos tipos de vinagre con mejores propiedades organolépticas y calidad.

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ABSTRACT

Introduction and Thesis motivation

The industrial elaboration of vinegar is performed from an alcoholic medium in which a mixed culture of acetic acid bacteria (AAB) is used to carry out a biotransformation process of ethanol into acetic acid. Although the acetification process is well known from a practical and technical point of view, there are still may fundamental aspects that have not been exhaustively studied, especially, those that ultimately control the activity and behavior of the complex microbiota responsible for the process. It is known that these microbial communities are mostly composed of a few species of AAB, strict aerobes and the main responsible for the aforementioned process (Gullo et al., 2014; Peters et al., 2017). However, minor fractions of both AAB and other microorganisms may coexist in the medium with the predominant species and contribute to the community function (Trček et al., 2016; Peng et al., 2021). Members of these microbiota and, especially AAB, because of their particular growing conditions based mainly on the requirement for a liquid medium rich in ethanol as a carbon source, homogeneous, and with constant dissolved oxygen supply, are difficult to isolate and cultivate outside these environments where they are normally developed (Mamlouk and Gullo, 2013; Gullo et al., 2014). In this context, the identification, as well as biodiversity and behavior studies of these microorganisms at a molecular level present several difficulties. These aspects not only imply a challenge to improve the understanding of scientific-basic concepts of these bacteria but hinder to control of the final quality of the obtained products, since it depends on the microbial composition, the raw material, and the operating conditions (Mas et al., 2014).

In this context, omics sciences currently offer multiple possibilities for the identification and characterization of microbial communities in their natural environment, without the need for isolation. In the field of vinegar production, existing studies are mainly focused on metagenomics and metabolomics of traditional vinegars, both those obtained through solid-state and surface fermentation, as well as molecular studies of concrete species (Xia et al., 2016; Peters et al., 2017; Wu et al., 2017; Zhu et al., 2018; Wang et al., 2021). This work, focused on the use of submerged culture as the production method and diverse omics tools, aims to contribute to expanding current knowledge in this research area.

Content of the Thesis

In the present Doctoral Thesis, the development of three acetification profiles was characterized and compared to study the evolution of system variables and the effect of the three raw materials used: a synthetic alcohol-based medium, a fine wine, and a craft beer, on the composition and activity of the microbiota responsible for the process. With this objective, different omics tools based on metaproteomics (LC-MS/MS), metagenomics (16S rRNA sequencing), protein fingerprinting (MALDI-TOF MS), and metabolomics (SBSE couple with GC-MS) were used, as well as submerged culture, working in a semi-continuous mode, for vinegar production at a pilot scale, trying to mimic industrial procedures.

First, the characterization of the acetification profiles of working raw materials, from a metaproteomic approach, allowed the description of the composition and behavior of the microbiota responsible for the process. The *Komagataeibacter* genus, represented mostly by the species *Komagataeibacter europaeus* and followed by other related species, conformed to the predominant microbiota. Additionally, this analysis revealed a minor fraction of microorganisms composed of typical genera of the Acetobacteraceae family, never described in vinegar to date. Subsequent metagenomics and protein fingerprinting studies confirmed the predominance of the *Komagataeibacter* genus and allowed the description of new bacterial genera in these media and even archaea groups. From the metaproteomics studies, quantitative analyses focused on the protein profile of the main species, *K. europaeus*, allowed for differentiating among the main biological processes that occur throughout the acetification cycle according to the variations in the activity of associated proteins. The results from the characterization and comparison of two acetification profiles of natural raw materials: fine wine and craft beer, allowed us to suggest a molecular strategy in which *K. europaeus* may ensure its survival through the use of nutrients present in each substrate. Based on this, the metabolization of acetic acid, from incomplete oxidation of ethanol, through the TCA cycle and other metabolic related pathways (pentose phosphate pathway and glycolysis), supplying biosynthetic precursors (amino acids and nucleic acids), as well as membrane mechanisms for acetic acid release, may be processes of biotechnological interest for the submerged vinegar production. Finally, a preliminary study at a metabolomic level led to the characterization of the "volatilome" and differentiation of key minor volatiles of both

the raw materials and their respective acetifications, allowing to establish significant differences, these being more evident between the synthetic and natural media.

Conclusion

The research conducted in this work has allowed for characterizing and confirming the composition of the microbiota present throughout three acetification profiles, with *K. europaeus* as predominant species followed by a less-abundant microorganisms fraction including closely related species, species from other typical AAB genera, groups of bacteria, other than AAB, and even archaea. From a quantitative point of view, it has been demonstrated that the use of diverse raw materials does not directly influence the composition of the microbiota, mostly common, but it influences the activity, behavior, and molecular strategies used throughout the acetification.

These findings may contribute to improving the existing current knowledge on the composition and role of the microbial communities responsible for the acetification, as well as expanding the use of new raw materials for the elaboration of vinegar through submerged culture. In turn, these achievements might lead to the improvement of the operating conditions for the obtention of new types of vinegar with improved organoleptic properties and quality.

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Section 1

INTRODUCTION

1. INTRODUCTION

1.1. The vinegar

1.1.1. The historical context of vinegar: origin and uses

Vinegar has its origin in ancient civilizations, concretely, the first testimony written on the use of vinegar comes from ancient Babylon, about 5,000 years ago, being employed as a food preservative. Vinegar was "discovered" fortuitously when undisturbed stored wine in the open-air turned spontaneously into vinegar (Andrés-Barrao and Barja, 2017). Probably, due to this phenomenon, known as "wine pitting", and its sour taste, vinegar has been considered historically as a byproduct with poor commercial interest. However, the numerous applications and benefits of vinegar have been disclosed by mankind throughout history. Hippocrates (460-377 BCE) recommended vinegar for cleaning ulcerations and for the treatment of sores (Johnston and Gaas, 2006). Long afterward, in the 10th century, Sung Tse implemented the use of vinegar as a hand-washing agent to prevent infections which led to an important development in the field of forensic medicine in China (Tan, 2005; Johnston and Gaas, 2006; Ho et al., 2017). In the 18th century, American medical practitioners used vinegar to treat many ailments including poison ivy, stomachache, high fever, and edema among others (Tan, 2005; Ho et al., 2017). Nowadays, vinegar is widely consumed all over the world both directly and included in a great variety of products including sauces, ketchup, and mayonnaise (Ho et al., 2017).

Although vinegar has been traditionally used as a flavoring and food preservative, several studies evidence its nutritional potential effects which can, directly, affect the health of consumers. This is due, in great part, to the healthy properties that acetic acid, the main constituent of vinegar, can exert on the human liver and gastrointestinal tract (Ali et al., 2018). As a consequence, some of the benefits of vinegar may include appetite stimulation, recovery from exhaustion, antioxidant activity, lower lipid content in blood, and regulation of blood pressure which, in turn, have an effect on biomarkers for several diseases such as obesity, cancer, diabetes, and hypertension among others, see Figure 1 (Honsho et al., 2005; Chang and Fang, 2007; Yamashita et al., 2007; Budak and Güzel-Seydim, 2010; Budak et al., 2011, 2014; Chou et al., 2015; Ali et al., 2018).

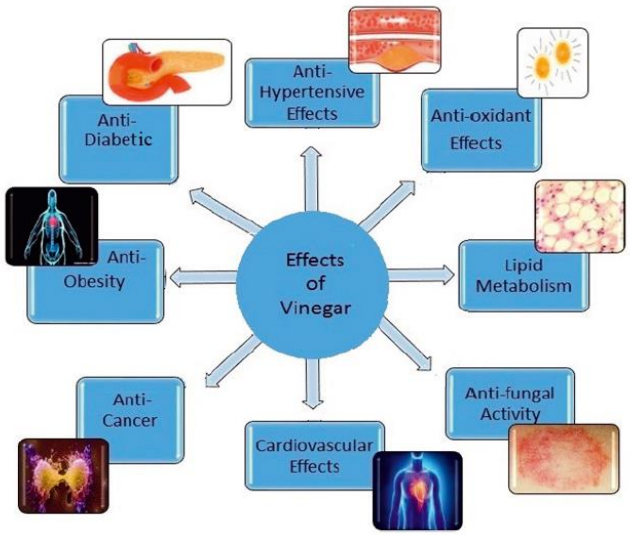

Figure 1. Effects of vinegar on human metabolism and several diseases. **Source**: adapted from Ali et al. 2018.

1.1.2. Definitions of vinegar

The definition of vinegar comes, etymologically, from the Latin term "*Vinum acre*" equivalent to "*sour wine*", however, in this meaning, its origin is not limited to wine, but it can be applied to any substrate containing fermentable sugars. According to the World Health Organization, as stated in its Codex Alimentarius Commission (Codex Alimentarius Commission, 1987), vinegar is a liquid that is fit for human consumption and produced, exclusively, from suitable products containing starch and/or sugars by double fermentation processes, alcoholic and acetic acid. Vinegar shall not contain more than 0.5% (v/v) ethanol and less than 50 g/L (w/v) acetic acid; stabilizers are not permitted for use according to European law. The European Union (UNE-EN 13188/AC:2002) defines vinegar as a product originated, exclusively, from double fermentation biological processes, alcoholic and acetic acid, of agricultural origin substances. This standard also indicates that the total acidity of vinegar shall not contain less than 45 g/L (w/v) and, concretely, less than 60 g/L (w/v) for wine vinegar, both calculated in terms of water-free acetic acid. The residual alcohol content must not exceed 0.5% (v/v) for standard vinegar and 1% (v/v) for wine vinegar. In Spain, the sanitary-technical regulation (Royal Decree 661/2012, 13th April) indicates that the total acetic acid content for wine vinegar must be at least 60 g/L (w/v), as well as 50 g/L (w/v) for the rest. The residual alcohol content may be a maximum of 0.5% (v/v), except for wine vinegar [1% (v/v)].

1.1.3. Varieties of vinegar

There are a great variety of vinegars around the world whose organoleptic properties are conferred by the starter microbial composition, raw material, and technical methods used for its production (Mas et al., 2014; Li et al., 2015b). Vinegar has an alcoholic origin, normally, coming from the processing of vegetables or fruits (Budak et al., 2014). In this way, several raw materials can be processed and used as acetification substrates including wines, spirits, cereal grains (rice wines and malts), and fruit juices, among others. Other raw materials of animal origin can be used such as whey or honey (Parrondo et al., 2009; Baena-Ruano, 2013; Ho et al., 2017; Lazim et al., 2019). This section will describe some of the most widely used varieties of vinegar in the world whose raw materials are specific to particular regions and confer on the final product exceptional organoleptic properties and high quality.

1.1.3.1. The Mediterranean vinegar: wine and balsamic vinegar

In Mediterranean countries, wine is the most used raw material due to the importance of grapevine cultivation in this region. Wine-producing countries are usually major vinegar-producing countries (Maestre et al., 2008). White and red wines allow producing a large part of total wine vinegars (Sellmer-Wilsberg, 2009). In Spain, there are three Protected Designations of Origin (PDOs): “Sherry Vinegar”, “Vinegar of Condado de Huelva”, and “Vinegar of Montilla-Moriles”, all of them located in Andalusia (Durán-Guerrero et al., 2021). Climate and soil factors of this region allow the growing of native varieties of grapes used for producing high-quality wines which confer on final vinegar exceptional organoleptic properties (Mas et al., 2014). The aging process by the method known as “Criaderas y Soleras” is one of the singularities of these vinegars which enhances, even more, their uniqueness (Lucena-Velasco, 2006). In northern Italy, traditional balsamic vinegar has two PDOs: “L'Aceto Balsamico Tradizionale di Modena” and “L'Aceto Balsamico Tradizionale di Reggio Emilia” (Giudici et al., 2009). From the native varieties of grapes grown in this region near Modena, a must is obtained which is subsequently cooked. The cooked must is then subjected to a simultaneous and spontaneous alcoholic and acetic acid fermentation, followed by a prolonged aging period of, at least, 12 years using barrels of different types of wood and sizes (Gullo and Giudici, 2008; Giudici et al., 2009).

1.1.3.2. Spirit vinegar

Spirit vinegar, also known as white vinegar, is obtained by the acetic acid fermentation of an alcohol medium coming from a previous alcoholic fermentation of an agricultural product containing fermentable sugars (Grierson, 2009). Spirit vinegar can reach the highest acidity levels [15-20% (w/v)] and although from a sensory point of view, its organoleptic profile is usually very poor, in quantitative terms, it is one of the most produced vinegars worldwide, mainly in Great Britain, Germany, and the eastern USA (Budak et al., 2014; Andrés-Barrao et al., 2016). For these reasons, spirit vinegar is used in studies that aim to achieve a high-yield acetification profile. Its main applications are as a cleaning product, seasoning, and food preservative (Budak et al., 2014).

1.1.3.3. Cereal vinegar

Traditional cereal vinegar has a long history with thousands of years of development and improvement that bind it to the Asian continent (Giudici et al., 2017; Zhang et al., 2019). These vinegars differ according to several factors such as the type of cereal and legumes used as raw material, the microbial composition of starter cultures, elaboration procedures, and aging times (Giudici et al., 2017). Among them, rice vinegar, obtained by acetic acid fermentation of rice wine "*sake*", is popular in Asian countries. In Japan, they are classified into polished rice vinegar "*Komesu*", unpolished rice vinegar "*Kurosu*", sake-less vinegar "*Kasuzu*", and other grain vinegars (Murooka et al., 2009). Different starchy substrates from each region are used for making some of the most famous Chinese vinegars including Shanxi aged vinegar, Zhenjiang aromatic vinegar, Sichuan bran vinegar, and Fujian *Monascus* vinegar (Chen et al., 2009; Xu et al., 2011; Zhu et al., 2018; Jiang et al., 2019). The particularities of processing the cereal vinegar include the use of solid-state fermentation (SSF), in which the starter culture is previously treated to allow the dominant microbiota to carry out the saccharification and subsequent alcohol fermentation of the grains, as well as to obtain a final product slightly milder and sweeter than Western vinegars (Chen et al., 2009; Wu et al., 2017).

1.1.3.4. Fruit vinegar

Fruit vinegar is usually elaborated as an alternative for the exploitation of existing fruit surpluses, thus reducing the economic and environmental impact produced by the fruit

industry (Luzón-Quintana et al., 2021). Although Asian countries were the first ones to become interested in fruits as raw materials, their use and study in other parts of the world have been increasing, see Figure 2A. The acidic nature of fruit vinegar and the high sensory impact of acetic acid on its organoleptic properties allow almost any type of fruit to be used for its elaboration. Among them, well-known cider vinegars are elaborated using apple juice through double alcoholic and acetic acid fermentation, especially in the United Kingdom, the United States, and Switzerland (Joshi and Sharma, 2009). Many other fruits have been explored for the elaboration of vinegars such as berry, persimmon, strawberry, pineapple, cherry, orange, mango, banana, and tomato in the last few years, see Figure 2B (Luzón-Quintana et al., 2021). Raw material processing is essential for the extraction of juice; crushing or pressing fruits are usually the most employed methods. Both traditional surface culture and submerged culture methods can be employed (Joshi and Sharma, 2009; Budak et al., 2011; Trček et al., 2016; Luzón-Quintana et al., 2021). Depending on the fruit used, the final product will have different nutritional composition; in the case of cider vinegar, the high polyphenols content of apples is responsible for its exclusive organoleptic properties such as a high astringency and viscosity as well as numerous health benefits (Joshi and Sharma, 2009; Budak et al., 2011, 2014; Ousaaid et al., 2021).

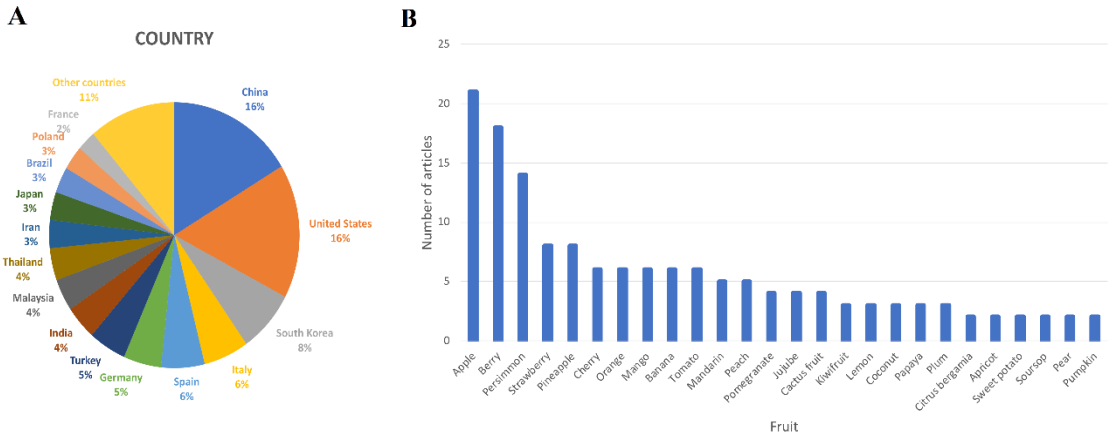

Figure 2A. Percent distribution of scientific articles on fruit vinegar published from 2015 to 2020, according to the country of the research groups (Source: Scopus). **Figure 2B.** Different fruits (other than grapes) used for the elaboration of vinegar for which two or more scientific articles about the technological process have been found in the literature from 1990 to 2020. **Source**: adapted from Luzón-Quintana et al. 2021.

1.1.4. Systems of vinegar production

Vinegar elaboration can be carried out by either solid-state fermentation (SSF) or liquid fermentation, which includes a set of techniques implemented in Western and European countries, mainly surface and submerged cultures. The submerged culture is one of the main systems to produce vinegar on an industrial scale (Gullo et al., 2014; Trček et al., 2016; Lee et al., 2017; Álvarez-Cáliz et al., 2021).

1.1.4.1. Traditional systems: solid-state fermentation and surface culture

SSF consists of a series of traditional techniques in which the microbiota responsible for the fermentation grows on substrates in the absence of free water (Gullo et al., 2014). These systems are very used in Asian countries to elaborate vinegar from grains (cereals) and to obtain high-quality vinegar after a period of aging (Wu et al., 2010; Xia et al., 2019). SSF includes three main biological processes: (1) starch liquefaction and saccharification, (2) alcohol fermentation, and (3) acetic acid fermentation. However, this method may be slower and low efficient than other techniques (Gullo et al., 2014).

Among the rest of traditional systems, the Orléans, Luxembourgish, and Schützenbach methods are the most known (Tesfaye et al., 2002; Mas et al., 2014; Bekatorou, 2019). The Orléans or French method is the main surface culture system, consisting of an old procedure based on the use of wooden barrels to elaborate vinegar. The substrate used consists of a mixed culture of wine and vinegar while acetic acid bacteria (AAB) are located on the surface forming a biofilm known as “the mother of vinegar” (Andrés-Barrao et al., 2011; Mas et al., 2014). The acetification and aging processes occur simultaneously to obtain a high-quality product, but this method is too slow and involves high production costs (Tesfaye et al., 2002; Raspor and Goranovič, 2008). The Luxembourgian and Schützenbach or German methods implement an immobilization system of AAB using supports made from wood shavings. This allows for increasing the contact surface between AAB and the acetification substrate, thus improving the oxygenation of the medium and the acetification yield (Llaguno, 1991; Baena-Ruano, 2013). Despite the high quality of vinegars obtained by these methods, they show some disadvantages as the difficulties of controlling the system's operational variables such as temperature, oxygen supply, as well as volatile compounds and ethanol losses through evaporation. In addition, the bioprocess is normally slowed down and the system does

not allow obtaining final products with acetic acid concentrations higher than 8-10% (w/v) (Llaguno, 1991; Tesfaye et al., 2002).

1.1.4.2. Submerged culture system

To industrialize vinegar production, the submerged culture was developed. Through this system, a submerged fermentation process takes place by which the ethanol content of raw materials such as spirits, wines, or juice fruits is oxidized to acetic acid by AAB under controlled stirring conditions (Gullo et al., 2014; Trček et al., 2016). This biotransformation is carried out in short periods (24-48 h) and it allows to obtain high acidity final products. This is mainly possible because of the efficiency of mass transfer and continuous vigorous aeration throughout the process (García-García et al., 2009, 2019). Some aspects that contribute to the high efficiency of this method are described as follows.

1.1.4.2.1. The bioreactor: acetator Frings

The current success of vinegar-making industries is undoubtedly given by the use of the acetator Frings developed by Hromatka and Ebner (2002) and marketed by Heinrich Frings GmbH and Co., Bonn, Germany, see Figure 3. These bioreactors have stainless steel tanks that can work with different volumes, from a pilot-scale (effective capacity of 8 L) up to an industrial scale volume (20,000-100,000 L) (Llaguno, 1991). They are equipped with coils as heat exchangers to maintain a constant temperature of 30-31 ºC and an efficient volatile recovery system by condensation of gasses; as a result, the losses of volatile compounds because of stripping are considerably minimized (De Ory et al., 2004; García-García et al., 2009; Gullo et al., 2014). But undoubtedly, the aeration system of these bioreactors confers on them a great part of their success. It consists of a turbine system that sucks air from the outside and releases it inside resulting in very fine air bubbles thus generating a homogeneous mixture with the culture medium (García-García et al., 2009; Fernández-Pérez et al., 2010; Gullo et al., 2014; Qi et al., 2014). Through this system, a higher oxygenation efficiency is reached thus obtaining higher acetification yields than using the traditional methods.

Figure 3. Bioreactor Frings on a pilot scale (effective capacity, 8 L) making wine vinegar. **Source**: own work.

1.1.4.2.2. Operating modes

Another fundamental aspect lies in the operating modes used for these bioreactors which, mainly, may work in a batch, semi-continuous, and continuous way. Although this choice depends on the specific purpose, which may comprise many factors, in general, a suitable environment for the development and activity of acetic acid bacteria must be ensured (García-García et al., 2009; Gullo et al., 2014). According to several authors, AAB may show high sensibility to different variables including ethanol concentration, acetic acid concentration, the total strength of the medium (sum of the previous two), temperature, and available dissolved oxygen (García-García et al., 2007; Baena-Ruano et al., 2010a, b; Santos-Dueñas et al., 2015; Jiménez-Hornero et al., 2020). In this sense, numerous studies have demonstrated that using a continuous mode, a maximum acidity level of 8-10% (w/v) can be achieved because a higher concentration or even a low ethanol content can affect the specific growth rate of AAB (Gullo et al., 2014). Furthermore, the batch mode normally implies lower productivity and additional difficulty in the preparation and maintenance of starter cultures for each cycle (Baena-Ruano, 2013).

The semi-continuous working mode has been mainly imposed for the industrial production of vinegar (De Ory et al., 2004; García-García et al., 2007, 2009; Gullo et al., 2014). In this method, each cycle is started by a loading phase that replenishes the reactor with a fresh medium to the working volume without exceeding a preset ethanol concentration. Then, an exhausting stage occurs depleting the ethanol in the culture broth to a preset extent. Finally, a part of the reactor is partially unloaded and the remaining volume is used as inoculum for the next cycle, see Figure 4 (Lee et al., 2017; Jiménez-Hornero et al., 2020). Working in this way, the operational variables are the initial concentration of ethanol in the culture medium, the concentration to which ethanol must be depleted for a cycle to be finished, the volume of the broth that is then unloaded, and the rate at which the bioreactor is loaded with fresh medium (García-García et al., 2007; Baena-Ruano et al., 2010a, b). Because AAB are highly sensitive to both ethanol and acetic acid, cell concentration and viability can be strongly affected by fermentation conditions (García-García et al., 2009, 2019). Therefore, an appropriate selection of the values of operational variables is essential for maintaining suitable ranges of both substrate and product concentrations and, in this way, the natural self-selection of the best-adapted AAB to the specific working medium is carried out (Jiménez-Hornero et al., 2020). This system also allows for obtaining high-strength vinegars that may reach high acidity levels [up to 15% (w/v)]. With the high demand for these products may be necessary to use dual-stage-high-strength processes in which two fermentation tanks are operated in a synchronous mode thus achieving acetic acid concentrations even above 20% (w/v) (Álvarez-Cáliz et al., 2021). In this way, the stressful environment to which AAB are subjected detracts from the overall acetification rate (García-García et al., 2009).

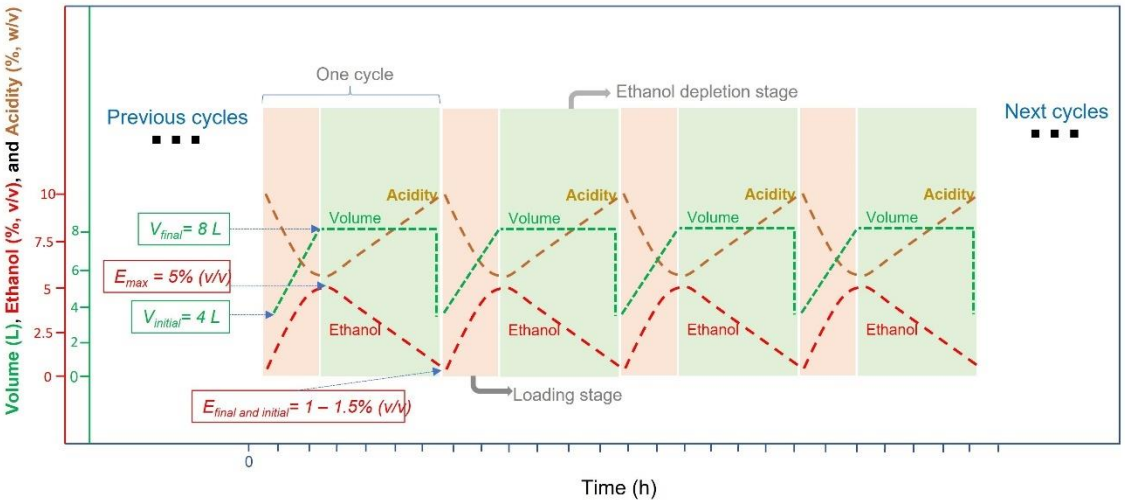

Figure 4. Submerged culture for vinegar production working in a semi-continuous mode. Each cycle of acetification starts by loading the tank to its working volume (8 L) without exceeding a preset ethanol concentration [5% (v/v)]. When ethanol concentration is depleted to 1.0-1.5% (v/v), 50% of the reactor content (4 L) is unloaded. This system is maintained for the following production cycles. **Source**: own elaboration.

1.1.4.2.3. Automation systems

Each operating mode, particularly the semi-continuous one, required control and monitoring because the particular fermentation conditions may induce variations in the development of the cycles even under identical conditions (García-García et al., 2009; Kalogianni et al., 2019). For this, the use of a monitoring system is necessary to obtain a constant recording of data of the main variables to be measured including the volume of the medium, the concentration of ethanol and dissolved oxygen in it, and temperature (García-García et al., 2007; Qi et al., 2014). Monitoring is usually performed by the Supervisory Control and Data Acquisition (SCADA) software which allows the setting of instructions to specific sensors controlled by signal acquisition modules (García-García et al., 2009; Baena-Ruano, 2013). This scheduling system also allows sampling at critical moments of the cycle, such as at specific points in the loading and unloading periods, by different measuring devices such as probes and transducers equipped with sensors that continuously monitor and register all values of each main aforementioned variable (Jiménez-Hornero et al., 2020). As an example, Figure 5 shows a pilot plant, on a laboratory scale, consisting of a Frings Acetator (8 L) working in a semi-continuous mode and equipped with an automation system that allows control of the main system variables.

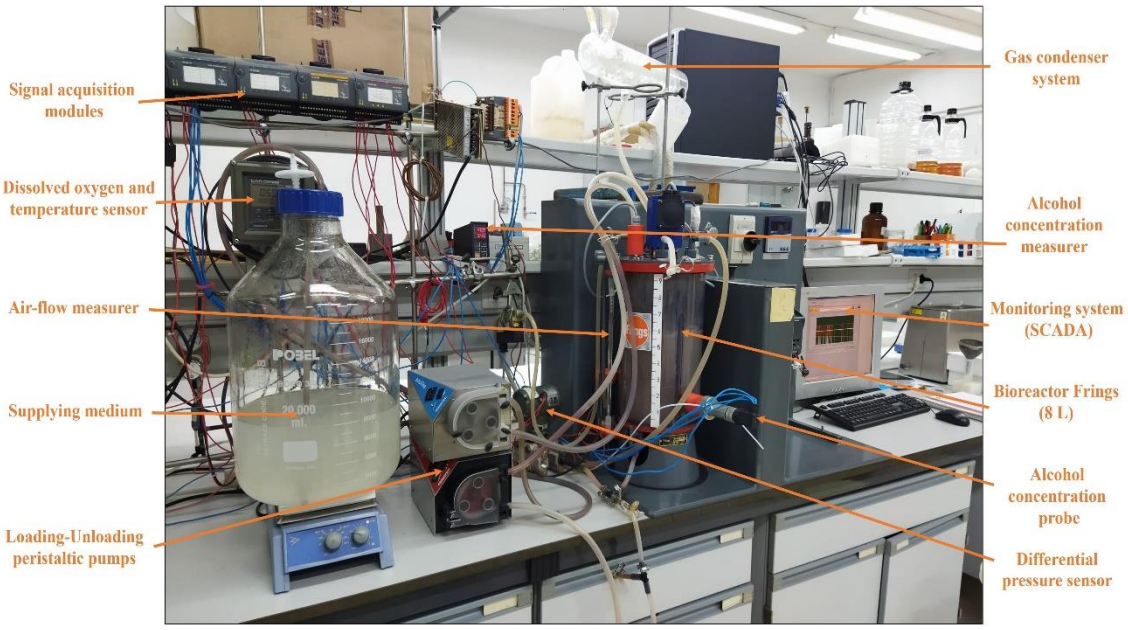

Figure 5. Pilot plant, on a laboratory scale, equipped with a Frings Acetator (8L) working in a semi-continuous mode and an automation system that controls the main variables of the system. **Source**: Biochemical Engineering Laboratory, Chemical Engineering Section, University of Córdoba, Spain.

1.2. Acetic acid bacteria

1.2.1. General characteristics of acetic acid bacteria

The vinegar elaboration would not be possible without the activity of the acetic acid bacteria (AAB), see Figure 6. These bacteria are Gram-negative or Gram-variable and their metabolism is strictly aerobic by using molecular oxygen (O_2) as the last electron acceptor. Despite this, some strains of *Acetobacter* and *Gluconobacter* may survive in a dormant state under low dissolved oxygen concentrations, as throughout the alcoholic fermentation in winemaking, being potentially reactivated during the wine clarification (Mas et al., 2014; Saichana et al., 2015; Jackson, 2020). AAB are catalase positive and oxidase negative, their optimum growing temperature usually ranges between 25-30 ºC, and their optimum growing pH is between 5-6.5 although many AAB do not present difficulties in growing at much lower pH levels, between 3-4 (Trček et al., 2015; Wang et al., 2015a; Gomes et al., 2018). The tolerance to low pH depends on parameters such as ethanol and acetic acid concentrations and oxygen availability (Baena-Ruano, 2013). Regarding their shape, most AAB are ellipsoidal or cylindrical, their size usually ranges between 0.4-1 μm wide and 0.8-4.5 μm long, and can be observed under the microscope alone, in pairs, or in aggregates and chains (Malimas et al., 2017).

These microorganisms constitute a very heterogeneous bacterial group whose cells are normally mobile with peritrichous or polar flagellation. In nature, AAB are found on substrates containing sugars and/or alcohols such as fruit juice, wine, cider, beer, and vinegar (Mas et al., 2014). On them, sugars and alcohols are incompletely oxidized thus producing organic acids such as acetic acid coming from ethanol, performed by the genera *Acetobacter* and *Komagataeibacter*, or gluconic acid coming from glucose, carried out by the genus *Gluconobacter* (Mamlouk and Gullo, 2013; Andrés-Barrao et al., 2016; Qiu et al., 2021). This ability of AAB is of great interest to the biotechnological industry; however, vinegar production is still the most extensively used industrial application.

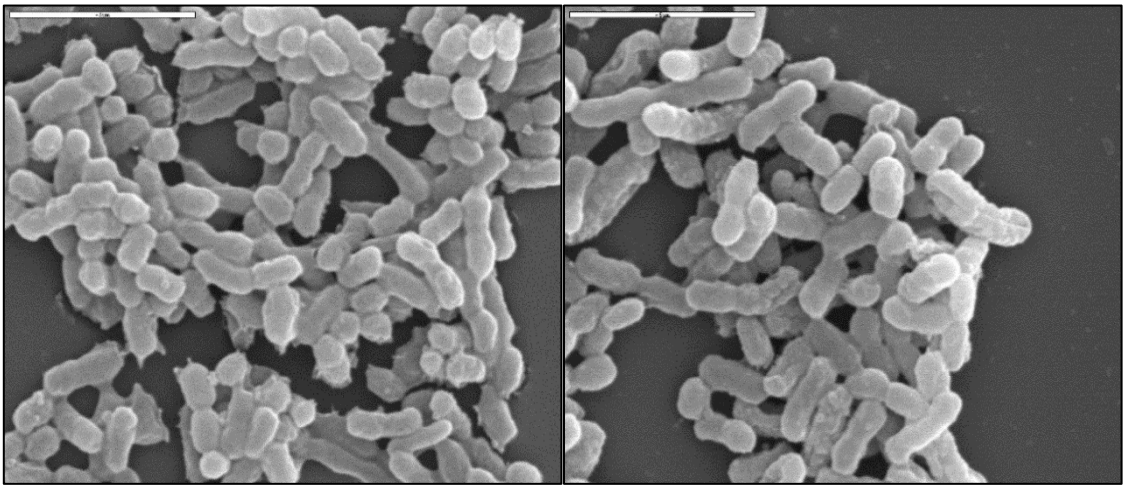

Figure 6. Images taken by scanning electron microscopy (SEM) of the acetic acid bacteria working inside the bioreactor used (Frings, 8 L). **Source**: own work.

1.2.2. Current taxonomy of acetic acid bacteria

Acetic acid bacteria are classified in the family Acetobacteraceae included in the order Rhodospirillales of the class Alphaproteobacteria. Acetobacteraceae consists of two groups, an acetous group and an acidophilic group based on ecological and phylogenic studies (Komagata et al., 2014). The former includes AAB, which share different a set of general features (see section 1.2.1.) and includes several genera: *Acetobacter*, *Asaia*, *Gluconacetobacter*, *Gluconobacter*, *Granulibacter*, and *Komagataeibacter* among many others. The acidophilic group has natures and origins physiologically and biochemically heterogeneous and includes other acidophilic and neutrophilic genera like *Acidiphilum* and *Roseomonas* among many others (Komagata et al., 2014).

Regarding the acetic acid bacteria group, *Acetobacter* was the first proposed genus (Skerman et al., 1980). In the 1960s, the taxonomy of AAB was significantly influenced by the chemotaxonomic study of the G + C content of DNA, quinone systems, cellular fatty acid composition, and DNA-DNA hybridization (Gillis and De Ley, 1980). Throughout time, four main genera of AAB (*Acetobacter*, *Gluconobacter*, *Gluconacetobacter*, and *Komagataeibacter*) were confirmed based on their membrane-bound dehydrogenases, which define their ethanol oxidation capabilities, and the type of respiratory coenzyme chain they contained (Yamada, 1983, Yamada et al., 2012). Through the development of polyphasic classification techniques that integrate several phenotypic, chemotactic, and genotypic data, new genera and species have been continuously reported (Cleenwerck and De Vos, 2008). Further, data from phylogenetic analysis based on 16S ribosomal RNA (rRNA) gene sequences have had a profound impact on the systematics of AAB, as well the rest of the genera of the family Acetobacteraceae (Komagata et al., 2014).

Currently, up to 47 genera and 207 species belonging to the family Acetobacteraceae have been identified according to Hördt et al. 2020 and the List of Prokaryotic names with Standing in Nomenclature (LPSN) database (Parte et al., 2020). From them, 20 genera and 108 species belong to AAB updated by the author from Qiu et al. 2021. Table 1 recompiles the most updated classification of the family Acetobacteraceae to date and highlights the acetic acid bacteria.

1.2.3. Metabolism of acetic acid bacteria

The molecular and biochemical aspects that define the metabolism of acetic acid bacteria are becoming more and more the target of many research works. In this section, a general and updated overview of the main AAB metabolic pathways, especially those related to the carbon sources assimilation including alcohols, sugars, and sugar alcohols for the production of organic acids, has been performed. It is worth noting that many other related metabolic pathways, partially or completely unknown, are being presently studied by several authors (Zheng et al., 2017; Li et al., 2019; Sankuan et al., 2021; Sriherfyna et al., 2021; Wang et al., 2021). A detailed review of these molecular strategies, mainly at an “omics level”, can be found in section 1.3.3.

Table 1. Current classification of the Acetobacteraceae family. Acetic acid bacteria are highlighted in bold. **Source**: adapted from the LPSN database.

Species	Reference	Species	Reference
Acetobacter aceti	Skerman et al., 1980	***Gluconobacter potus***	Sombolestani et al., 2021
Acetobacter cerevisiae	Cleenwerck et al., 2002	***Gluconobacter roseus***	Malimas et al., 2008b
Acetobacter cibinongensis	Lisdiyanti et al., 2002	***Gluconobacter sphaericus***	Malimas et al., 2008c
Acetobacter conturbans	Sombolestani et al., 2020	***Gluconobacter thailandicus***	Tanasupawat et al., 2004
Acetobacter estunensis	Lisdiyanti et al., 2000	***Gluconobacter vitians***	Sombolestani et al., 2021
Acetobacter fabarum	Cleenwerck et al., 2008	***Gluconobacter wancherniae***	Yukphan et al., 2010
Acetobacter fallax	Sombolestani et al., 2020	***Granulibacter bethesdensis***	Greenberg et al., 2006
Acetobacter farinalis	Tanasupawat et al., 2011	*Humitalea rosea*	Margesin and Zhang 2013
Acetobacter garciniae	Yukphan et al., 2021	***Komagataeibacter diospyri***	Naloka et al., 2020
Acetobacter ghanensis	Cleenwerck et al., 2007	***Komagataeibacter europaeus***	Yamada et al., 2012
Acetobacter indonesiensis	Lisdiyanti et al., 2000	***Komagataeibacter intermedius***	Yamada et al., 2012
Acetobacter lambici	Spitaels et al., 2014a	***Komagataeibacter kakiaceti***	Yamada 2014
Acetobacter lovaniensis	Lisdiyanti et al., 2000	***Komagataeibacter kombuchae***	Yamada et al., 2012
Acetobacter malorum	Cleenwerck et al., 2002	***Komagataeibacter medellinensis***	Yamada 2014
Acetobacter musti	Ferrer et al., 2016	***Komagataeibacter melaceti***	Marič et al., 2020
Acetobacter nitrogenifiens	Dutta and Gachhui 2006	***Komagataeibacter melomenusus***	Marič et al., 2020
Acetobacter oeni	Silva et al., 2006	***Komagataeibacter nataicola***	Yamada et al., 2012
Acetobacter okinawensis	Iino et al., 2012	***Komagataeibacter oboediens***	Yamada et al., 2012
Acetobacter orientalis	Lisdiyanti et al., 2002	***Komagataeibacter rhaeticus***	Yamada et al., 2012
Acetobacter orleanensis	Lisdiyanti et al., 2000	***Komagataeibacter saccharivorans***	Yamada et al., 2012
Acetobacter oryzoeni	Baek et al., 2020	***Komagataeibacter sucrofermentans***	Yamada et al., 2012
Acetobacter oryzifermentans	Kim et al., 2018a	***Komagataeibacter swingsii***	Yamada et al., 2012
Acetobacter papayae	Iino et al., 2012	***Komagataeibacter xylinus***	Yamada et al., 2012
Acetobacter pasteurianus	Skerman et al., 1980	***Kozakia baliensis***	Lisdiyanti et al., 2002
Acetobacter persici	Iino et al., 2012	*Lichenicoccus roseus*	Pankratov et al., 2020
Acetobacter pomorum	Sokollek et al., 1998	*Lichenicola cladoniae*	Noh et al., 2020

Table 1. Continued.

Species	Reference	Species	Reference
Acetobacter sacchari	Vu al., 2019	*Muricoccus roseus*	Kämpfer et al., 2003
Acetobacter senegalensis	Ndoye et al., 2007	***Neoasaia chiangmaiensis***	Yukphan et al., 2005
Acetobacter sicerae	Li et al., 2014	***Neokomagataea tanensis***	Yukphan et al., 2011
Acetobacter suratthaniensis	Pitiwittayakul et al., 2016	***Neokomagataea thailandica***	Yukphan et al., 2011
Acetobacter syzygii	Lisdiyanti et al., 2002	***Nguyenibacter vanlangensis***	Vu et al., 2013
Acetobacter thailandicus	Pitiwittayakul et al., 2015	***Novacetimonas cocois***	Brandão et al., 2022
Acetobacter tropicalis	Lisdiyanti et al., 2000	***Novacetimonas hansenii***	Brandão et al., 2022
Acidibrevibacterium fodinaquatile	Muhadesi et al., 2019	***Novacetimonas maltaceti***	Brandão et al., 2022
Acidicaldus organivorans	Johnson et al., 2006	***Novacetimonas pomaceti***	Brandão et al., 2022
Acidiphilium acidophilum	Hiraishi et al., 1998	*Oecophyllibacter saccharovorans*	Chua et al., 2020
Acidiphilium angustum	Wichlacz et al., 1986	*Paracraurococcus ruber*	Saitoh et al., 1998
Acidiphilium cryptum	Harrison 1981	*Rhodopila globiformis*	Imhoff et al., 1984
Acidiphilium iwatense	Okamura et al., 2015	*Rhodovarius crocodyli*	Chen et al., 2020
Acidiphilium multivorum	Wakao et al., 1994	*Rhodovarius lipocyclicus*	Kämpfer et al., 2004
Acidiphilium organovorum	Lobos et al., 1986	*Rhodovastum atsumiense*	Okamura et al., 2009
Acidiphilium rubrum	Wichlacz et al., 1986	*Roseicella frigidaeris*	Khan et al., 2019
Acidisoma cellulosilyticum	Mieszkin et al., 2021	*Roseococcus microcysteis*	Kim et al., 2021
Acidisoma sibiricum	Belova et al., 2009	*Roseococcus suduntuyensis*	Boldareva et al., 2009
Acidisoma silvae	Mieszkin et al., 2021	*Roseococcus thiosulfatophilus*	Yurkov et al., 1994
Acidisoma tundrae	Belova et al., 2009	*Roseomonas aeriglobus*	Lee and Jeon 2018
Acidisphaera rubrifaciens	Hiraishi et al., 2000	*Roseomonas aerilata*	Yoo et al., 2008
Acidocella aluminiidurans	Kimoto et al., 2010	*Roseomonas aerofrigidensis*	Hyeon and Jeon 2017
Acidocella aminolytica	Kishimoto et al., 1995	*Roseomonas aerophila*	Kim et al., 2013
Acidocella aquatica	Okamoto et al., 2017	*Roseomonas aestuarii*	Venkata-Ramana et al., 2010
Acidocella aromatica	Jones et al., 2013	*Roseomonas algicola*	Kim et al., 2020
Acidocella facilis	Kishimoto et al., 1995	*Roseomonas alkaliterrae*	Dong et al., 2014

Table 1. Continued.

Species	Reference	Species	Reference
Acidomonas methanolica	Urakami et al., 1989	*Roseomonas aquatica*	Gallego et al., 2006
Ameyamaea chiangmaiensis	Yukphan et al., 2009	*Roseomonas arctica*	Qiu et al., 2016
Asaia astilbis	Suzuki et al., 2010	*Roseomonas arcticisoli*	Kim et al., 2016b
Asaia bogorensis	Yamada et al., 2000	*Roseomonas bella*	Zhang et al., 2020
Asaia krungthepensis	Yukphan et al., 2004a	*Roseomonas cervicalis*	Rihs et al., 1993
Asaia lannensis	Malimas et al., 2008a	*Roseomonas coralli*	Li et al., 2021a
Asaia platycodi	Suzuki et al., 2010	*Roseomonas deserti*	Subhash and Lee 2018
Asaia prunellae	Suzuki et al., 2010	*Roseomonas eburnea*	Wang et al., 2016
Asaia siamensis	Katsura et al., 2001	*Roseomonas elaeocarpi*	Damtab et al., 2016
Asaia spathodeae	Kommanee et al., 2010	*Roseomonas fluminis*	Ko et al., 2018
Belnapia arida	Molina-Menor et al., 2021	*Roseomonas frigidaquae*	Kim et al., 2009
Belnapia moabensis	Reddy et al., 2006	*Roseomonas gilardii*	Rihs et al., 1993
Belnapia mucosa	Molina-Menor et al., 2021	*Roseomonas globiformis*	Fang et al., 2018
Belnapia rosea	Jin et al., 2012	*Roseomonas harenae*	Deng et al., 2020
Belnapia soli	Jin et al., 2013	*Roseomonas hellenica*	Rat et al., 2021
Bombella apis	Yun et al., 2017	*Roseomonas hibiscisoli*	Yan et al., 2017
Bombella favorum	Hilgarth et al., 2021	*Roseomonas lacus*	Jiang et al., 2006
Bombella intestini	Li et al., 2015a	*Roseomonas ludipueritiae*	Sánchez-Porro et al., 2009
Bombella mellum	Hilgarth et al., 2021	*Roseomonas mucosa*	Han et al., 2003
Caldovatus sediminis	Habib et al., 2017	*Roseomonas musae*	Nutaratat et al., 2013
Commensalibacter intestini	Roh et al., 2008	*Roseomonas nepalensis*	Chaudhary and Kim 2017
Craurococcus roseus	Saitoh et al., 1998	*Roseomonas oleicola*	Wu et al., 2021
Crenalkalicoccus roseus	Ming et al., 2016	*Roseomonas oryzae*	Ramaprasad et al., 2015
Dankookia rubra	Kim et al., 2016a	*Roseomonas oryzicola*	Chung et al., 2015
Elioraea rosea	Lee and Whang 2020	*Roseomonas pecuniae*	Lopes et al., 2011
Elioraea tepidiphila	Albuquerque et al., 2008	*Roseomonas ponticola*	Yin et al., 2021

Table 1. Continued.

Species	Reference	Species	Reference
Elioraea thermophila	Habib et al., 2020	*Roseomonas radiodurans*	Kim et al., 2018b
Endobacter medicaginis	Ramírez-Bahena et al., 2013	*Roseomonas rhizosphaerae*	Chen et al., 2014
Entomobacter blattae	Guzman et al., 2021	*Roseomonas riguiloci*	Baik et al., 2012
Gluconacetobacter aggeris	Nishijima et al., 2013	*Roseomonas rubea*	Lee and Whang 2022
Gluconacetobacter asukensis	Tazato et al., 2012	*Roseomonas rubra*	Subhash et al., 2016
Gluconacetobacter azotocaptans	Fuentes-Ramírez et al., 2001	*Roseomonas sediminicola*	He et al., 2014
Gluconacetobacter diazotrophicus	Yamada et al., 1997	*Roseomonas selenitidurans*	Hou et al., 2020
Gluconacetobacter dulcium	Sombolestani et al., 2021	*Roseomonas soli*	Kim and Ka 2014
Gluconacetobacter entanii	Schüller et al., 2000	*Roseomonas stagni*	Furuhata et al., 2008
Gluconacetobacter johannae	Fuentes-Ramírez et al., 2001	*Roseomonas suffusca*	Subhash and Lee 2017
Gluconacetobacter liquefaciens	Yamada et al., 1997	*Roseomonas terrae*	Yoon et al., 2007
Gluconacetobacter sacchari	Franke et al., 1999	*Roseomonas terricola*	Kim et al., 2017
Gluconacetobacter takamatsuzukensis	Nishijima et al., 2013	*Roseomonas tokyonensis*	Furuhata et al., 2013
Gluconacetobacter tumulicola	Tazato et al., 2012	*Roseomonas vastitatis*	Zhao et al., 2020
Gluconacetobacter tumulisoli	Nishijima et al., 2013	*Roseomonas vinacea*	Zhang et al., 2008
Gluconobacter aidae	Yukphan et al., 2020	*Roseomonas wenyumeiae*	Tian et al., 2019
Gluconobacter albidus	Yukphan et al., 2004b	*Roseomonas wooponensis*	Lee et al., 2015
Gluconobacter cadivus	Sombolestani et al., 2021	*Rubritepida flocculans*	Alarico et al., 2002
Gluconobacter cerevisiae	Spitaels et al., 2014b	***Saccharibacter floricola***	Jojima et al., 2004
Gluconobacter cerinus	Yamada and Akita 1984	*Siccirubricoccus deserti*	Yang et al., 2017
Gluconobacter frateurii	Mason and Claus 1989	*Siccirubricoccus phaeus*	Li et al., 2021b
Gluconobacter japonicus	Malimas et al., 2009a	***Swaminathania saitolerans***	Loganathan and Nair 2004
Gluconobacter kanchanaburiensis	Malimas et al., 2009b	***Swingsia samuiensis***	Malimas et al., 2013
Gluconobacter kondonii	Malimas et al., 2007	***Tanticharoenia aidae***	Vu et al., 2016
Gluconobacter morbifer	Roh et al., 2008	***Tanticharoenia sakaeratensis***	Yukphan et al., 2008
Gluconobacter oxydans	De Ley 1961		

1.2.3.1. Biotransformation of ethanol to acetic acid

The overall oxidative biological reaction that defines the biotransformation of ethanol into acetic acid can be represented as follows:

$$C_2H_5OH + O_2 \longrightarrow CH_3COOH + H_2O \quad \Delta H^o = -520\ KJ \times mol^{-1}$$

AAB are chemoorganotrophs microorganisms that use ethanol coming from a medium of alcoholic origin as a carbon source. The genera *Acetobacter* and *Komagataeibacter* usually show a higher ethanol preference although other AAB groups may show a preference for other carbon sources (Gullo et al., 2014; Andrés-Barrao et al., 2016). This biotransformation consists of an incomplete oxidation reaction of two steps. First, alcohol dehydrogenase (ADH) binds to pyrroloquinoline quinone (PQQ) to oxidize the ethanol into acetaldehyde. Next, acetaldehyde is oxidized to acetic acid by membrane-bound aldehyde dehydrogenase (ALDH); both enzymes are located on the periplasmic side of the inner cell membrane (Adachi et al., 1980; Ameyama and Adachi, 1982). Oxidized nicotinamide adenine dinucleotide (NAD^+) and nicotinamide adenine dinucleotide phosphate ($NADP^+$), located in the cytoplasm, may be used as coenzymes by NAD-ADH, NAD-ALDH, and NADP-ALDH (Sriherfyna et al., 2021; Qin et al., 2022). The inner acetic acid can be completely oxidized by the acetyl-CoA synthase, which led the input of acetyl-CoA in the TCA cycle and here, up to CO_2 and H_2O providing energy (ATP) and detoxifying the cell (Matsushita et al., 2016; He et al., 2022). Other organic acids such as lactic, pyruvic, malic, succinic, citric, and fumaric may be similarly metabolized (Mamlouk and Gullo, 2013). Because of the strictly aerobic metabolism of AAB, the ADH-PQQ and ALDH complexes are closely linked to the respiratory chain, which transfers reducing equivalents from donor substrates to ubiquinone (UB). Then, electrons from the reduced UB named ubiquinol (UBH_2), are transferred to the final electron acceptor, oxygen (O_2), by terminal ubiquinol oxidases (UOX) producing H_2O, see Figure 7 (He et al., 2022; Qin et al., 2022).

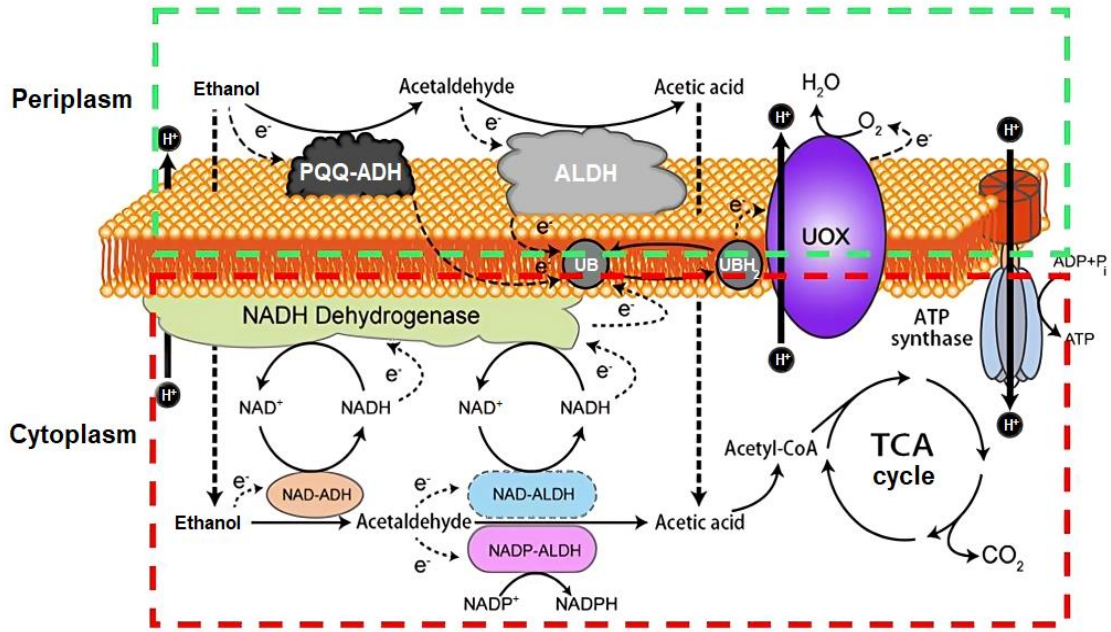

Figure 7. Incomplete oxidation reaction of ethanol into acetic acid both (1) at the cell membrane level (green box): PQQ-ADH, PQQ-dependent alcohol dehydrogenase; ALDH, membrane-bound aldehyde dehydrogenase; UB, ubiquinone; UBH_2, ubiquinol; UOX, ubiquinol oxidase; and (2) the cytoplasm level (red box): NAD-ADH, NAD-dependent alcohol dehydrogenase; NAD-ALDH, NAD-dependent aldehyde dehydrogenase; NADP-ALDH, NADP-dependent aldehyde dehydrogenase. ATP, energy; TCA cycle, Tricarboxylic Acid Cycle. **Source**: adapted from He et al. 2022.

The ADH complex of most AAB is composed of three subunits although also may contain two subunits in any species (Yakushi and Matsushita, 2010). Subunit I (72-78 kDa), encoded by the gene *adhA*, is a catalytic component containing a PQQ and a heme C moiety. Subunit II (44-45 kDa), encoded by the gene *adhB*, is a membrane-anchoring and ubiquinone-reducing component possessing three heme C moieties; these both subunits participate in the intramolecular electron transport to the terminal UB. Subunit III (20 kDa), encoded by the gen *adhS*, which has no prosthetic group, facilitates the association of subunits I and II to the membrane and acts as a molecular chaperone for folding and/or maturation of subunit I (Mamlouk and Gullo, 2013; Qin et al., 2022). Several authors have related a high ADH stability and activity with a high tolerance and production of acetic acid, mainly, in species from the current genus *Komagataeibacter* (Trček et al., 2007; Andrés-Barrao et al., 2016). The ALDH complex is composed of two or three subunits depending on the AAB species and acts as an operon. Although its optimum pH ranges between 4-5, the oxidation of acetaldehyde to

acetate may be catalyzed at lower pH values. ALDH is highly sensitive to low oxygen concentrations and the presence of ethanol in the medium (Mamlouk and Gullo, 2013).

1.2.3.2. Carbohydrates oxidation metabolism

AAB can metabolize different carbohydrates as carbon sources, mainly glucose, but also arabinose, fructose, galactose, mannose, ribose, sorbose, and xylose (Mamlouk and Gullo, 2013). Most AAB have been characterized by non-functional glycolysis because of the absence of phosphofructokinase enzyme; therefore, the pentose phosphate pathway (PPP) is the main metabolic route of AAB to oxidize the glucose available in the medium by the catalytic activity of the enzymes glucose-6-P dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGD) providing metabolic precursors such as ribulose-5-phosphate, generating NADPH + H^+, and energy (Adler et al., 2014; García-García et al., 2017; Yin et al., 2017). Among AAB, several species from *Gluconobacter* are glucose-preference and several *Gluconobacter oxydans* strains also exhibit the ability to oxidize glucose to gluconic acid via glucono-δ-lactone forming D-gluconate. This oxidation reaction occurs in the periplasm by a membrane-bound pyrroloquinoline quinone-dependent glucose dehydrogenase (PQQ-GDH) located on the outer side of the cytoplasmic membrane. D-gluconate can be further oxidized rapidly to ketogluconates such as 2-ketogluconate (2-KGA), 5-ketogluconate (5-KGA), and 2,5-diketogluconic acid (2,5-DKGA) both in the periplasm and cytoplasm by different oxidizing enzymes, see Figure 8 (Bringer and Bott, 2016; García-García et al., 2017; Kiefler et al., 2017). Glucose, gluconic acid, and ketogluconates can be assimilated by these bacteria thus obtaining biomass, energy, and acidifying the medium, possibly, as a part of their metabolic strategy to prevail over other glucose-like microorganisms (García-García et al., 2017). Final products of PPP and Entner Doudoroff pathway (EDP) may be completely oxidized to CO_2 and H_2O by *Acetobacter*, *Gluconacetobacter*, and *Komagataeibacter* spp. using the TCA cycle when carbon source of the medium is exhausted but not by *Gluconobacter* spp., which show a non-functional TCA cycle (Mamlouk and Gullo, 2013; Kiefler et al., 2017).

AAB also exhibit the ability to oxidize several sugar alcohols such as glycerol, D-mannitol, and D-sorbitol among others, being especially remarkable in winemaking the use of glycerol as a carbon source producing dihydroxyacetone (DHA) by the activity of some oxidizing enzymes, mainly glycerol dehydrogenase, and providing energy (ATP)

via gluconeogenesis. Strains from *Acetobacter pasteurianus*, *Gluconobacter oxydans*, and *Komagataeibacter xylinus* are some of the most studied regarding this oxidative pathway (Mamlouk and Gullo, 2013; La China et al., 2018).

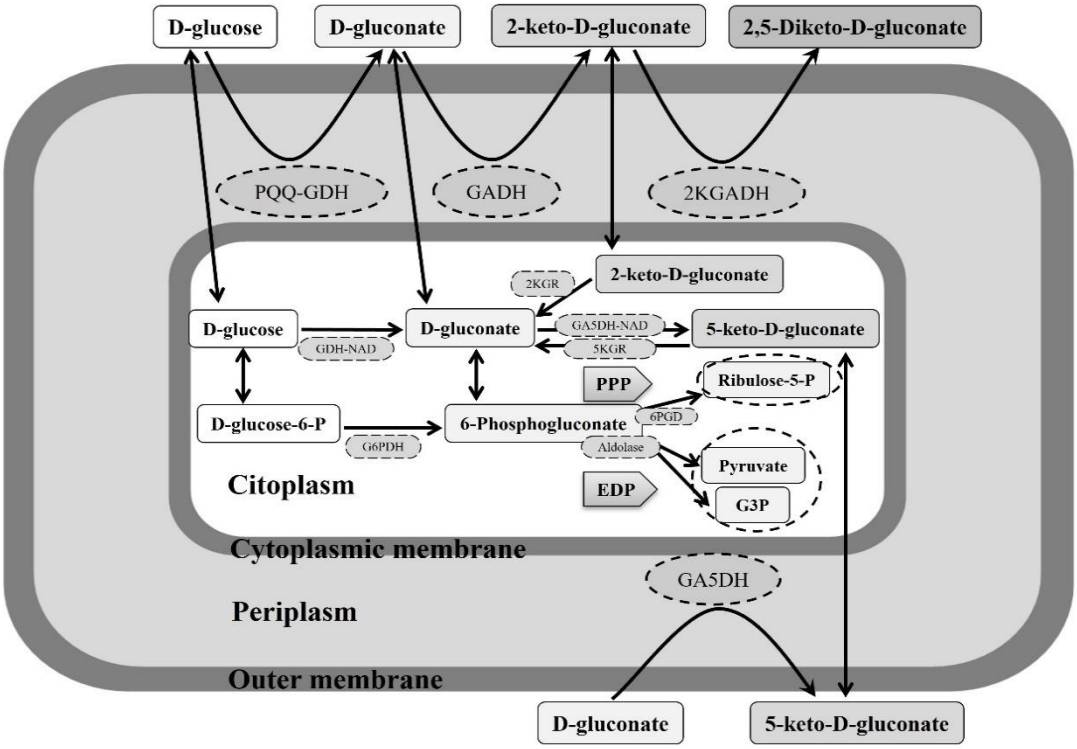

Figure 8. Glucose metabolism in *Gluconobacter*. Cell membrane enzymes: PQQ-GDH, PQQ-dependent D-glucose dehydrogenase; GADH, FAD-dependent D-gluconate 2-dehydrogenase; 2KGADH, FAD-dependent 2-keto-D-gluconate dehydrogenase; GA5DH, PQQ-dependent D-gluconate 5-dehydrogenase. Cytoplasm enzymes: GDH-NAD, NADP-dependent D-glucose dehydrogenase; GA5DH-NAD, NADP-dependent D-gluconate 5-dehydrogenase; 2KGR, 2-keto-D-gluconate reductase; 5KGR, 5-keto-D-gluconate reductase; G6PDH, glucose-6-phosphate dehydrogenase; 6PGD, 6-phosphogluconate dehydrogenase. Compounds: G3P, glyceraldehyde 3-phosphate. Pathways: PPP, pentose phosphate pathway; EDP, Entner Doudoroff pathway. **Source**: adapted from García-García et al. 2017.

1.2.4. Biotechnological applications of acetic acid bacteria

AAB are the main microorganisms responsible for vinegar production, but they are also used in different applications very useful in biotechnology which are increasingly being investigated. Other foods can be produced as the result of the activity of AAB, as is the case of kombucha, a traditional beverage obtained by fermenting sugary tea with a symbiotic culture of acidophilic yeasts and bacteria including acetic acid bacteria (AAB) and lactic acid bacteria (LAB) immobilized in a microbial cellulose biofilm

known as tea fungus (Gomes et al., 2018; Villareal-Soto et al., 2018). First, yeasts transform sugars from tea into organic acids, ethanol, and CO_2. Then, AAB may synthesize different compounds such as acetic acid (*Acetobacter aceti*, *Acetobacter pasteurianus*), gluconic acid (*Gluconobacter oxydans*), and bacterial cellulose (*Komagataeibacter xylinus*) due to the high biodiversity of AAB. This product is becoming more and more popular because of its probiotic characteristics as a treatment of gastrointestinal disorders as well as improving general health and increasing longevity attributed due to its acidic composition and high phenolic antioxidant content (Ayed et al., 2017; Gomes et al., 2018; Villareal-Soto et al., 2018). Another product, gluconic acid, is industrially obtained by the oxidation of glucose by several AAB, mainly *Gluconobacter oxydans*. Gluconic acid improves the sensory properties of food products and may also be used as an additive and preservative by the food industry. Due to its role in the aromatic profile of foods, gluconic acid has been proposed as a quality parameter of food products (Mounir et al., 2016; Gomes et al., 2018). Gluconic acid is used in the pharmaceutical industry as gluconates of divalent metals, which function as mineral supplements to treat some diseases (Cañete-Rodríguez et al., 2016). The high oxidative capability of strains of *Gluconobacter* is also exploited to convert sugar alcohols as in the case of D-sorbitol to L-sorbose, an important intermediate in industrial production of L-ascorbic acid (vitamin C), an antioxidant very used in the food industry (Mamlouk and Gullo, 2013).

Among biotechnological applications of AAB, the production of bacterial cellulose has attracted interest in recent years because of its extreme purity, unlike plant-derived cellulose, thus representing a promising alternative for many industries (Gomes et al., 2018). Among its multiple applications, bacterial cellulose is employed as a gelling, stabilizing, and thickening agent in foods, heart medicine, pharmacy, and skin repair in wound healing and burn treatments (Shi et al., 2014; Mohammadkazemi et al., 2015; Ullah et al., 2016). *K. xylinus* is the most commonly used species of AAB because of its capability to produce high amounts of bio-cellulose from different carbon and nitrogen sources and involves different enzymes such as glucose kinase, phosphoglucomutase, UDP-glucose pyrophosphorylase, and membrane-bound cellulose synthase (Kuo et al., 2016). Besides bio-cellulose, AAB may produce other microbial exopolysaccharides, such as levans, dextran, acetan, mannan, and gluconacetan with important industrial applications (Gomes et al., 2018; Anguluri et al., 2022).

1.3. Omics sciences applied to vinegar-producing microbiota

1.3.1. Methods of molecular identification

The particular growing conditions and metabolic characteristics of AAB hinder their isolation by traditional methods in solid media, outside the environments in which they carry out their activity fully (Fernández-Pérez et al., 2010; Mamlouk and Gullo, 2013). This occurs especially from fermented beverages such as industrially produced vinegar within bioreactors since here, the microbiota involved requires proper concentrations of substrate (ethanol) and product (acetic acid), low pH, a constant oxygen supply, and aeration of the medium (García-García et al., 2019). This phenomenon, known as viable but non-culturable (VBNC) state, limits the study of the richness and biodiversity, probably ignoring key species that compose these microbiota inhabiting aggressive media (Mamlouk and Gullo, 2013). Traditionally, AAB have been identified according to several morphological, biochemical, and physiological criteria, however, current identification methods are focused on the global analysis of biological macromolecules (DNA, RNA, and proteins) and metabolites in a cell, tissue, organism, or population at critical moments and under specific conditions (Cleenwerck and De Vos, 2008; Porras-Agüera, 2020). In this way, the "omics sciences" emerge as an alternative to solve many of the problems present by traditional methods. In this section, the main molecular techniques used throughout the time for the identification and typing of AAB are described according to the taxon identified (genus, species, and strain) and the macromolecule selected (DNA, RNA, protein, and metabolite).

1.3.1.1. Genomics and metagenomics

The use of molecular techniques for the identification of AAB began with DNA-based techniques more than 25 years ago. After the first assays focused on selective extraction of nucleic acids such as plasmid profiling (Teuber et al., 1987; Mariette et al., 1991) and DNA-DNA hybridization (Boesch et al., 1998), the PCR-based systems were developed. PCR amplification of specific regions on the 16S rRNA gene and restriction fragment length polymorphism (RFLP)-PCR of the same gene, let to identify microbial populations in vinegar at the genus and species levels (Sievers et al., 1998; Ruiz et al., 2000). The digestion using restriction enzymes such as *taql* and *rsal* allowed discrimination between AAB genera, although the high conservation of 16S rRNA gene

required the use of 16S-23S rRNA intergenic spacer region (ITS) with a higher variability than functional sequences for differentiating under a species level (Ruiz et al., 2000; González et al., 2006). Other PCR-based methods include first, the amplification of specific regions on other genes such as *adhA* in *K. europaeus* and *nifH*/*nifD* in nitrogen-fixing AAB (Loganathan and Nair, 2004; Trček, 2005; Dutta and Gachhui, 2006); and second, a combination of other techniques including enterobacterial repetitive intergenic consensus (ERIC)-PCR, repetitive extragenic palindromic (REP)-PCR, $(GTG)_5$-rep-PCR, quantitative real-time PCR (qPCR), nested PCR, random amplified polymorphic DNA (RAPD)-PCR, and amplified fragment length polymorphism (AFLP) which several authors have used to identify, typing, and some to enumerate species and strains of AAB mainly in wine and vinegar microbiota (Ruiz et al., 2000; González et al., 2005, 2007; De Vuyst et al., 2008; Cleenwerck et al., 2009; Fernández-Pérez et al., 2010; Vegas et al., 2010; Li et al., 2014). Although these techniques have been described as rapid methods for the taxonomic grouping of AAB, an accurate identification is only possible based on a polyphasic approach. In this sense, the electrophoresis-based techniques act by the separation of amplified fragments of the 16S rRNA gene according to their mobility under denaturing conditions (Lopez et al., 2003). Both denaturing gradient gel electrophoresis (DGGE)-PCR and denaturing high-performance liquid chromatography (DHPLC) have been used to group the main genera of AAB involved in traditional (De Vero and Giudici, 2008) and submerged vinegar production (Trček et al., 2016). Both approaches are useful for monitoring structural changes of fermented food microbiota, however, due to the small size of DNA fragments, only identification to genus level is possible. For this reason, Andrés-Barrao et al. 2016 used DGGE-PCR along with housekeeping sequencing of genes (*dnaK*, *groEL*, *rpoB*) and multi-locus sequence typing (MLST) to build a detailed phylogenetic tree of *Komagataeibacter* strains making high-acid spirit vinegar.

Recently, metagenomics and massive sequencing appear as new technologies that allow the analysis of the genomic DNA or RNA from all organisms of a microbial population (Rizo et al., 2018). These next-generation sequencing (NGS) tools provide a great deal of information about the gene content and function allowing both the identification and classification of microbiomes, rebuilding metabolic routes, and comparing experimental conditions to find differential microbial composition, abundance, and function (Mayo et al., 2014; Escobar-Zepeda et al., 2015). Amplicon metagenomics (pyrosequencing and

Illumina) of specific genes or genome regions was applied to identify and quantify metagenomes of AAB in different types of vinegar produced by surface (Peng et al., 2015; Valera et al., 2015) and submerged systems (Trček et al., 2016). Finally, "shotgun" metagenomics implements the use of the sequencing data to infer potential metabolic functions encoded by the genomes of the community members under study through the assembly of the sequence reads followed by gene prediction and even discovering whole genomes of VBNC microorganisms (Smukowski-Heil et al., 2018; Verce et al., 2019). In vinegar, this technology was first used for the analysis of the microbiota of cereal vinegar to reveal the flavor metabolic network (Wu et al., 2017).

1.3.1.2. Proteomics and metaproteomics

The DNA within the cells contains the genetic map of the whole organism, however, the cellular phenotype can only be detected by studying proteins. Proteins are responsible for carrying out one or more specific functions within cells and because of their high activity and sensitivity to environmental changes, their study throughout fermentation dynamics is increasingly widespread (Elviri and Mattarozzi, 2012; Rizo et al., 2018). Proteomics is the analysis of the entire set of proteins produced by a cell or organism allowing us to identify them and quantify their abundance thus offering a precise picture of what is occurring in crucial moments and under specific conditions of a biological process (Pischetsrieder and Baeuerlein, 2009; Rizo et al., 2018). Analysis of complex microbiota, such as AAB inhabiting vinegar, is improved through metaproteomic approaches that let the study of the composition and function of multiple proteomes belonging to different species or strains with high throughput (Heyer et al., 2017).

The first protein-based technique used for the analysis of the vinegar microbiota was two-dimensional electrophoresis (2DE) which separates complex mixes of proteins first, by isoelectric focusing (IEF) and second, according to their molecular weight (SDS-PAGE) using polyacrylamide gels (Elviri and Mattarozzi, 2012). Lasko et al. 1997 studied protein patterns in response to acetate stress in two acetate-resistant species of *Acetobacter*. A few years later, proteins for acetic acid response in *A. aceti* and their relationship with the TCA cycle as a strategy of its assimilation were determined (Nakano et al., 2004, 2006; Nakano and Fukaya, 2008). Although 2DE provided remarkable molecular advances for improving vinegar production, the trouble of the inherent lack of reproducibility between gels led to the development of differential gel

electrophoresis (DIGE) consisting of the labeled of two samples, each with a different fluorescent dye (Cy3-NHS, Cy5-NHS), before running them on the same gel (Minden, 2012). 2D-DIGE has allowed identifying differentially expressed proteins in the proteome of *A. pasteurianus* and metaproteome of *Komagataeibacter* spp. producing spirit vinegar (Andrés-Barrao et al., 2012, 2016).

Since all these molecular methods require extensive sample manipulation and intensive work, new approaches have been developed for rapid identification of bacteria (Trček and Barja, 2015). One of them, matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS), allows performing a rapid routine identification for a large number of bacteria samples obtaining a unique mass spectrum composed of several peaks corresponding to high-abundance soluble proteins. This results in a protein profile for each bacteria that allow for differentiation among genera, species, and strains (Trček and Barja, 2015; Gomes et al., 2018). MALDI-TOF MS has been described as a quick and reliable method for the identification of AAB involved in the industrial production of vinegar and beer spoilage (Andrés-Barrao et al., 2013; Wieme et al., 2014). In recent years, mass spectrometry (MS) technology has been combined with liquid chromatography (LC) leading to LC-MS, an analytical method sensitive, selective, and accurate consisting of the physical separation and mass-based detection of proteins, peptides, other macromolecules, and metabolites (Malachová et al., 2018; Lasch et al., 2020). By adding a second mass analyzer, both working in tandem (LC-MS/MS), "shotgun" metaproteomics technology emerged as a powerful tool for the fast identification of thousands of proteins from a metaproteome by analyzing complex mixtures of peptides resulting from their proteolytic digestion without a prior separation by electrophoresis and providing a wider dynamic range and protein coverage (Zhang et al., 2018; Roux-Dalvai et al., 2019). Although previous works have used free-label LC-MS/MS and isobaric tags for relative and absolute quantitation (iTRAQ) in the AAB field (Xia et al., 2016; Yin et al., 2017), "shotgun" metaproteomics by LC-MS/MS method for the study of a whole vinegar microbiota has been developed, to our knowledge, for the first time in the current Doctoral Thesis.

1.3.1.3. Metabolomics

Vinegar is mainly the result of numerous metabolic reactions in which the microbiota present are involved. This fact leads to the release of several metabolites (sugars, sugar

alcohols, amino acids, carboxylic acids, fatty acids, and volatile compounds) which comprise a metabolomic profile essential for the organoleptic properties of the final product (Pinu et al., 2016). Metabolomics is a recent approach that is often applied along with (meta)genomics and/or (meta)proteomics to relate macromolecules (genes, proteins, etc.) belonging to the microorganisms present in the medium with their corresponding produced metabolites (Patti et al., 2012; Rizo et al., 2018). Two types of approaches are used in metabolomic studies: untargeted and targeted analyses; however, the instrumental techniques for most of them consist of a previous separation of the metabolites from the samples by chromatography followed by their identification and quantification using MS according to the mass-to-charge (m/z) ratio (Patti et al., 2012; Pinu et al., 2016). For the analysis of different metabolomic vinegar profiles, above all balsamic and cereal vinegar, gas chromatography(GC)-MS (Pinu et al., 2016; Zhu et al., 2018), high-performance liquid chromatography(HPLC)-MS (Cocchi et al., 2002; Sanarico et al., 2003), capillary electrophoresis(CE)-MS (Lee et al., 2019), and nuclear magnetic resonance (NMR) spectrometry (Caligiani et al., 2007) have been widely used. GC-MS is one of the most mature technologies in metabolomics; it allows the identification and simultaneous analysis of hundreds of metabolites with high-resolution capability and sensitivity thus obtaining comprehensive metabolite profiles of fermented products. However, this method usually requires chemical derivatization for non-volatile metabolites (Villas-Bôas et al., 2005; Smart et al., 2010). For the extraction of volatiles, stir bar sorptive extraction (SBSE) with polydimethylsiloxane (PDMS) coating let to reduce the disadvantages of other extraction systems. This method does not use solvents, is simple, fast, highly sensitive, and shows suitable limits for the quantification and detection of volatile compounds in vinegar (Durán-Guerrero et al., 2006, 2007).

1.3.1.4. Databases and software for raw data analysis in omics sciences

After identification and/or quantification of the different macromolecules or compounds coming from the microorganisms inhabiting vinegar, to know their microbial composition and behavior on their natural media, the raw data obtained must be processed by using specific databases or software according to each omic science. Currently, the technologies that support not only the omics sciences procedures but also the subsequent raw data manipulation are continuously updated, improved, and

developed mainly in terms of efficiency and accuracy (Porras-Agüera, 2020). The choice of suitable tools for raw data processing and bioinformatic analyses is essential for the success of an experimental design working with omics sciences. Here, some of the most currently used databases or software in the omics sciences field are described, see Table 2.

Table 2. Compilation of the main databases, software, and tools used in the raw data processing mainly focused on the field of bacterial omics sciences. **Source**: own work.

Tool	Accession link	Omics science	Description
BLAST	https://blast.ncbi.nlm.nih.gov/Blast.cgi	(Meta)genomics and (meta)proteomics	Comparison of nucleotides or protein sequences
QIIME2	https://qiime2.org/	(Meta)genomics	Treatment metagenomic data and interactive visualization
LPSN	https://lpsn.dsmz.de/	(Meta)genomics	Taxonomic classification of prokaryotic microorganisms
Proteome Discoverer	—	(Meta)proteomics	MS raw data analysis
Uniprot	https://www.uniprot.org/blast/	(Meta)proteomics	Database of protein sequence and functional information. GO Term analysis
MaxQuant	https://www.maxquant.org/	(Meta)proteomics	Quantitative proteomics for high-resolution analysis. MS raw data treatment
Perseus	https://maxquant.net/perseus/	(Meta)proteomics	Proteins quantification, interaction, and PTM
STRING	https://string-db.org/	Proteomics	Protein-protein interactions and functional associations
KEGG	https://www.genome.jp/kegg/	Genomics	Collection of genome databases, enzymatic routes, and chemical compounds
BioCyc	https://biocyc.org/	Genomics and proteomics	Collection of genome databases, metabolic routes, and chemical compounds
MetaboAnalyst	https://www.metaboanalyst.ca/	Metabolomics and multi-omics	Metabolomic and other omics data analysis. Statistics
RStudio	https://www.rstudio.com/	Multi-omics	Raw data analysis. Programming, bioinformatics

1.3.2. Microbial biodiversity throughout the acetification process

The microbiota composition inhabiting vinegar has a crucial role in the organoleptic properties and quality of the final product. Omics sciences have certainly facilitated the

understanding of the microbial biodiversity throughout the acetification process by using different approaches such as metagenomics (Trček et al., 2016; Peters et al., 2017; Wu et al., 2017; Peng et al., 2021), transcriptomics (Sakurai et al., 2011; Wang et al., 2021), proteomics (Andrés-Barrao et al., 2012; Zhang et al., 2015; Xia et al., 2016; Zheng et al., 2017), metaproteomics (Andrés-Barrao et al., 2016), and metabolomics (Zhu et al., 2018; Jiang et al., 2019; Zhang et al., 2019). These studies have allowed identifying the microorganisms involved in these biotransformations as well as describing their behavior both under different operating conditions and media thus helping to elucidate the key role of the vinegar-making microbiota.

The microbial composition of vinegar is highly dependent on the starting inoculum, raw material, and production system (Mas et al., 2014; Li et al., 2015b). *Acetobacter* and *Komagataeibacter* are usually the main AAB responsible for the acetification process because of their high oxidative capabilities to transform ethanol into acetic acid, although other minor fractions of microorganisms might coexist with the best-adapted ones (Gullo et al., 2014; Wang et al., 2015a; Peng et al., 2021). Species of *Acetobacter* are usually damaged when the acetic acid concentration reaches 7-8% (w/v) with a maximum of 9-10% (w/v), so they are widely found in wine, cereal, and balsamic vinegar elaborated by traditional methods and early stages of those produced by submerged culture or low-acid vinegar, like cider vinegar [4.0-9.0% (w/v)] and wine vinegar [4.5-10% (w/v)]. *A. pasteurianus* is usually the most widely found species of this genus (Gullo et al., 2009; Gullo et al., 2014; Zhang et al., 2015) although *A. aceti*, *A. malorum*, and *A. pomorum* have been also detected in some of the aforementioned media, see Table 3 (Gullo and Giudici, 2008; Andrés-Barrao et al., 2013). In low-acid vinegar, the presence of bacteria other than AAB, such as lactic acid bacteria (*Lactobacillus*, *Leuconostoc*, *Oenococcus*, and *Pediococcus*) has been reported (Trček et al., 2016; Zhu et al., 2018).

Species of *Komagataeibacter* (main of them relocated from *Gluconacetobacter*), which can resist 15-20% (w/v) acetic acid, are highly predominant in submerged cultures including spirit vinegar and late stages of most white and red wine vinegar (Fernández-

Table 3. Main species, including acetic acid bacteria and lactic acid bacteria, that prevail in the microbiota of different types of vinegar according to the raw material used and production method. The acidity range (%, w/v) between which each vinegar is usually elaborated is included. **Source**: own work.

	Submerged culture production				**Traditional culture production (surface and solid-state)**		
Microorganism	Spirit vinegar	Wine vinegar	Cider vinegar	Fruit vinegar	Wine vinegar	Balsamic vinegar	Cereal vinegar
	10-20% (w/v)	4.5-10% (w/v)	4-9% (w/v)	4-9% (w/v)	4-7.5% (w/v)	4.5-7.5% (w/v)	4-7% (w/v)
Acetobacter	-	*A. pasteurianus*	*A. pasteurianus*	*A. pasteurianus*	*A. pasteurianus*	*A. pasteurianus*	*A. pasteurianus*
	-	*A. aceti*	*A. pomorum*	*A. malorum*	*A. aceti*	*A. aceti*	*A. aceti*
	-	-	-	-	-	*A. malorum*	-
Komagataeibacter	*K. europaeus*	*K. europaeus*	*K. europaeus*	*K. europaeus*	*K. europaeus*	*K. europaeus*	*K. europaeus*
	K. oboediens	*K. oboediens*	*K. intermedius*	*K. intermedius*	*K. intermedius*	*K. hansenii*	*K. medellinensis*
	K. intermedius	*K. intermedius*	*K. hansenii*	*K. rhaeticus*	*K. hansenii*	*K. xylinus*	*K. xylinus*
	-	*K. hansenii*	*K. xylinus*	*K. xylinus*	*K. rhaeticus*	-	-
	-	-	-	-	*K. xylinus*	-	-
Gluconacetobacter	*Ga. entanii*	-	-	-	-	-	-
Lactic acid bacteria	-	-	*Lactobacillus*	*Lactobacillus*	-	-	*Lactobacillus*
	-	-	*Oenococcus*	-	-	-	*Pediococcus*
	-	-	-	-	-	-	*Leuconostoc*

Pérez et al., 2010; Gullo et al., 2014; Andrés-Barrao et al., 2016). Because of their tolerance to low acidity levels [7-9% (w/v)], several strains have been also shown to participate in the acetification profiles of cider, other fruits, and even traditional vinegars (Fernández-Pérez et al., 2010; Fu et al., 2014; Trček et al., 2016). *K. europaeus* has been described as the main suitable AAB for the industrial production of vinegar due to its particular growing conditions (Trček et al., 2007; Andrés-Barrao et al., 2011) although other *Gluconacetobacter* (*Ga. entanii*) and *Komagataeibacter* species (*K. hansenii*, *K. intermedius*, *K. medellinensis*, *K. oboediens*, *K. rhaeticus*, and *K. xylinus*) are present in different types of vinegar, see Table 3 (Boesch et al., 1998; Schüller et al., 2000; Fernández-Pérez et al., 2010; Trček et al., 2016; Peters et al., 2017).

The biodiversity of the microbiota decreases with the increase in the concentration of acetic acid in the medium and operating conditions that control bioreactors cause these media to be even more selective since few species show the growing conditions that allow them to adapt and survive (Vegas et al., 2010; Gullo et al., 2014). However, recent studies highlight the presence of a minor fraction of microorganisms trying to coexist with the better-adapted species and contributing to the stability of the microbial community (Trček et al., 2016; Peng et al., 2021).

1.3.3. Key molecular strategies throughout the acetification process

The behavior of the microorganisms which participate in the industrial elaboration of vinegar may be influenced by the chemical features of the raw material, the production system, and operating conditions (Mas et al., 2014). Although the metabolism of these microbiota is driven by the incomplete oxidation reaction of ethanol into acetic acid, there are many other molecular strategies at both the cytoplasmic and membrane level for the adaptation and survival of the community members to the conditions imposed by the medium (Wang et al., 2015a; Xia et al., 2016; Peng et al., 2021; Qiu et al., 2021). Because the basic assimilative metabolism of the AAB has been described in section 1.2.3., here, a compilation of the main associated processes or pathways used by the microbiota as strategies, mainly focused on acetic acid throughout vinegar production, has been performed. The analyses of these strategies, some of them partially or completely unknown, have been approached mainly from an omics perspective.

1.3.3.1. Strategies on the cytoplasm: biosynthetic and stress-related processes

Acetic acid bacteria are available to produce a variety of macromolecules (amino acids, proteins, nucleic acids, and lipids) and metabolites (alcohols, sugar alcohols, esters, and other aromatic compounds) throughout acetification (Li et al., 2016; Zhu et al., 2018). Ammonium is a key nitrogen source used in the biosynthesis of amino acids, proteins, nucleotides, and volatile compounds (Gobert et al., 2019). Amino acids are synthesized through L-glutamine and L-glutamate both being nitrogen sources that are self-regulated according to the cell requirements (Yin et al., 2017; Sankuan et al., 2021). AAB may use their high nitrogen recovery capability to transform continuously nitrogen sources like proteins, nucleic acids, and apoptotic cells into ammonium and amino acids to replace the cell material losses throughout the submerged acetification (Álvarez-Cáliz et al., 2012; Kuypers et al., 2018). The deamination process consists of the switch of L-glutamine into L-glutamate by a glutaminase (YbaS) with the release of gaseous ammonia (NH_3) thus increasing the content of acid products (H^+, NH_4) at the end of acetification (Lu et al., 2013; Trček, 2015). In this sense, acetolactate synthase (Als) is a key enzyme related to the formation of branched-chain amino acids (BCAA) from pyruvate that may provide NH_3 and energy to neutralize this increase of final acid products and support intracellular pH balance, see Figure 9 (Santiago et al., 2012; Andrés-Barrao et al., 2016; Yin et al., 2017).

The biosynthesis of proteins is one of the most highlighted metabolic pathways in AAB throughout acetification. Ribosomal proteins and others ensuring an accurate translation process often undergo a decrease in their activity when the concentration of acetic acid increases at the final stages of the acetification (Ibba and Söll, 2000; Rubio-Gómez and Ibba, 2020). A negative effect of the acidity increase on ribosome integrity and protein biosynthesis has been described in proteomic and transcriptomic approaches (Andrés-Barrao et al., 2012; Xia et al., 2016; Wang et al., 2021). However, the activity of proteins that regulates the translation by recycling and inactivating ribosomes, such as ribosome recycling factor (RRF) and hibernation promoting factor (HPF), increases under high-acidity conditions. Then, several stress-related proteins, such as heat shock proteins 60 GroES, 10 GroEL and molecular chaperones DnaJ, DnaK, GrpE, and ClpB, have been described as protectors to prevent the protein denaturation and refolding under stress conditions (Hartl and Hayer-Hartl, 2002; Okamoto-Kainuma et al., 2002;

Hirokawa et al., 2005; Matzov et al., 2019). Studies performed in *A. pasteurianus* detailed an up-regulation of these proteins when the acidity was increased, so possible regulation of the formation and folding of proteins as a molecular strategy against acetic acid stress is proposed, see Figure 9 (Andrés-Barrao et al., 2012; Wang et al., 2015b; Xia et al., 2016).

It is also worth noting that throughout a submerged vinegar fermentation and, especially, those working in a semi-continuous mode, the microbiota is subjected to sudden changes in volume, substrate, and product concentration that trigger constants biotransformations. These oxidation reactions, performed under a continuous aeration condition, may generate a variety of toxic compounds and reactive oxygen species (ROS) in the cellular cytoplasm of AAB (Okamoto-Kainuma et al., 2008). Catalase (KatE), superoxide dismutase (SodB), ferredoxin (FdxA), glutaredoxin (GrxC), and bacterioferritin (Bfr), among many other oxidoreductases and cofactors (NADH/NAD^+ and NADPH/$NADP^+$), have been described as up-regulated proteins under acetic acid stress in different species of AAB, see Figure 9 (Xia et al., 2016; Sriherfyna et al., 2021). Redox homeostasis may be a valuable strategy and a crucial metabolic pathway to control submerged vinegar fermentation.

1.3.3.2. The tricarboxylic acid cycle (TCA)

The TCA cycle was one of the first metabolic pathways whose enzymes were shown to be associated with inner acetic acid assimilation (Nakano et al., 2004, 2006; Nakano and Fukaya, 2008). Cytoplasmic acetic acid can be completely oxidized to CO_2 and H_2O providing energy (ATP) and detoxifying the cell by the well-known overoxidation reaction (Matsushita et al., 2016). Acetyl-CoA synthase (Acs) catalyzes the conversion of acetate into acetyl-CoA and its input into the TCA cycle when the ethanol source in the medium is exhausted to promote secondary growth (Ramírez-Baena et al., 2013). *Acetobacter*, *Gluconacetobacter*, and *Komagataeibacter* spp. may use the TCA cycle but not *Gluconobacter* spp., which lacks some enzymes showing a non-functional TCA cycle (Mamlouk and Gullo, 2013; Kiefler et al., 2017). Proteomic and genomic analyses revealed that three genes (*aarA*, *aarB*, and *aarC*) are influenced by acetic acid stress and their deletion causes acid resistance lost in *A. aceti* 1023 (Fukaya et al., 1990). *AarA* and *aarC* genes, which encode citrate synthase (AarA) and succinyl-CoA transferase (AarC) respectively, were some of the first acetic acid resistance determinants (Mullins et al.,

2008). AarC is produced by *Acetobacter* and *Komagataeibacter* replacing succinyl-CoA synthetase (SucCD) in *Acetobacter* spp. classified into the *A. pasteurianus* group (Azuma et al., 2009; Mullins and Kappock, 2012). Aconitate hydratase (AcnA), another TCA cycle enzyme, was up-regulated when growing *A. aceti* in a 1% EtOH medium, and its overexpression increased the acetic acid resistance of the strain (Nakano et al., 2004). Proteomic and metaproteomic analyses of *A. pasteurianus* [4% (w/v)] and *Komagataeibacter* spp. [> 10% (w/v)] respectively, for submerged vinegar production, revealed up-regulated enzymes of the TCA cycle under high-acid conditions including citrate synthase (AarA), aconitate hydratase (AcnA), isocitrate dehydrogenase NAD^+ (Icd), succinate dehydrogenase (SdhA), fumarate hydratase (FumA/C), and succinyl-CoA transferase (AarC) among others (Andrés-Barrao et al., 2012, 2016). Considering that AAB must cope with continuous changes in the ethanol, acetic acid, and cell concentrations throughout submerged acetification, the TCA cycle may be used for assimilating inner acetic acid coming from ethanol, supplying energy and biosynthetic precursors, see Figure 9. The TCA cycle participates in the strategy that confers to the vinegar microbiota its inherent resistance to living and thriving in its natural aggressive media within bioreactors (Adler et al., 2014; Andrés-Barrao et al., 2016; Qiu et al., 2021).

1.3.3.3. Acetic acid resistance mechanisms on the cell membrane

The increase of acetic acid concentration in the medium may trigger modifications on the cell membrane morphology in AAB. *Acetobacter* and *Komagataeibacter* are acetic acid-producing species commonly found in the vinegar industry, but the latter shows higher acid resistance than the former (Qiu et al., 2021). *Acetobacter* spp. can be classified according to their surface shape into R (rough cell surface) and S (smooth cell surface), being the first related to a pellicle polysaccharide formation (Deeraksa et al., 2005). *A. pasteurianus* R strains have demonstrated a higher capability production and tolerance of acetic acid than S strains and intracellular acetic acid content of the latter is 3 or 4 times higher than that of the R strains thus showing a higher diffusion of acetate molecules into the cell of S strains due to the absence of the aforementioned pellicle (Kanchanarach et al., 2010). In *Komagataeibacter* spp., a lack of capsular polysaccharides (CPS) layer has been reported during the industrial production of vinegar, conversely to *Acetobacter* spp. Ethanol and acetic acid should diffuse freely

through the outer membrane throughout acetification, so the absence of the CPS layer may favor the exchange of metabolites between the cellular inner and the medium, thus enhancing industrial vinegar production (Andrés-Barrao et al., 2016). CPS may not be involved in the acetic acid resistance of *Komagataeibacter* but probably are in yield enhancement.

Regarding cell membrane composition, *Komagataeibacter* strains (*K. europaeus*) show higher phosphatidylcholine (PC) content than those of *Acetobacter* thus becoming the main phospholipid of the cell membrane, particularly in presence of acetic acid in the medium, see Figure 9. Likewise, non-polar glycolipids content also increased under these conditions which could result in the strengthening of the cell hydrophobic layer (Trček et al., 2007). Sphingolipids' content may also increase through dihydroceramide, whose synthesis has been directly related to acetic acid tolerance and the stability of the PQQ-ADH enzyme in *Acetobacter malorum* during vinegar production (Ogawa et al., 2010). *Komagataeibacter* spp. exhibit higher levels of hopanoids, particularly tetrahydroxybacteriohopane (THBH), which contributes to the stabilization of the cell membrane at high ethanol concentration and has been also related to the acetic acid resistance in AAB (Matsushita et al., 2016; Nakano and Esibuya, 2016). Alterations in the fatty acids' composition have been also described through the attenuation of the flux of the fatty acid pathway and subsequently reduce the total lipids content. The downregulation of two effector proteins in *A. pasteurianus*, FabD and FabG, involved in the biosynthesis and elongation of fatty acids under acetic acid stress is clear evidence of it (Xia et al., 2016). However, two genes involved in the increase of the proportion and chain length of unsaturated fatty acids, *des* and *cfa*, were activated in a *K. hansenii* strain under acetic acid stress which could indicate different strategies among AAB to adapt their membrane composition to the conditions imposed by the medium, see Figure 9 (Li et al., 2019).

On the other hand, proteins and enzymatic complexes located in the cell membrane may contribute to the molecular strategy of the vinegar-producing microbiota. Although well-known membrane-bound ADH-PQQ and ALDH system that carried out the acetic acid generation from ethanol has been described in section 1.2.3.1., it is worth noting that the activity of their enzymes has been also described. ADH-PQQ enzymes usually show high quantification throughout acetification although can exhibit more unstable

behavior as a consequence of the shift of the carbon source from glucose to ethanol or the growing phase in some AAB strains (Quintero et al., 2009; Andrés-Barrao et al., 2012). However, ADH-PQQ relevance in this transformation is usually greater than that of membrane-bound ALDH (Wang et al., 2021). Because the role of cytoplasmic enzymes (ADH-NAD and ALDH-NADP) is the conversion of the cytoplasmic ethanol, their activities during the acetification on the cell membrane are completely inhibited (Yakushi and Matsushita, 2010; Gullo et al., 2014; Qiu et al., 2021). Acetic acid can be released to the periplasm by an efflux pump proton motive force-dependent and ATP-binding cassette (ABC) transporters. A putative ABC transporter in *A. aceti*, named AatA, was associated with acid resistance acting as an efflux pump for acetic acid release (Matsushita et al., 2005; Nakano et al., 2006). Comparative genomic analysis demonstrated that species of *Komagataeibacter* contain more genes encoding putative ABC transporter proteins than *Acetobacter* (Wang et al., 2015b). This correlation may indicate that ABC transporters are directly associated with acetic acid resistance (Qiu et al., 2021). The outer membrane may also contribute to cell membrane function. The outer membrane protein (OMP) family acts as permeable porins of small solutes and maintains the stability of the outer membrane structure (Confer and Ayalew, 2013). OmpA, OmpH, OmpW, and OsmC were implied in the balance of nutrient uptake and resistance to the toxicity of molecular stressors in response to the high acidity in *A. pasteurianus* and *K. europaeus*. However, there are important differences in the behavior of these proteins and their corresponding coding genes between AAB genera throughout acetification, thus unraveling possible diverse strategies contributing to acetic acid resistance (Andrés-Barrao et al., 2012; Xia et al., 2016; Wang et al., 2021). These molecular strategies at the cell membrane level are described in Figure 9.

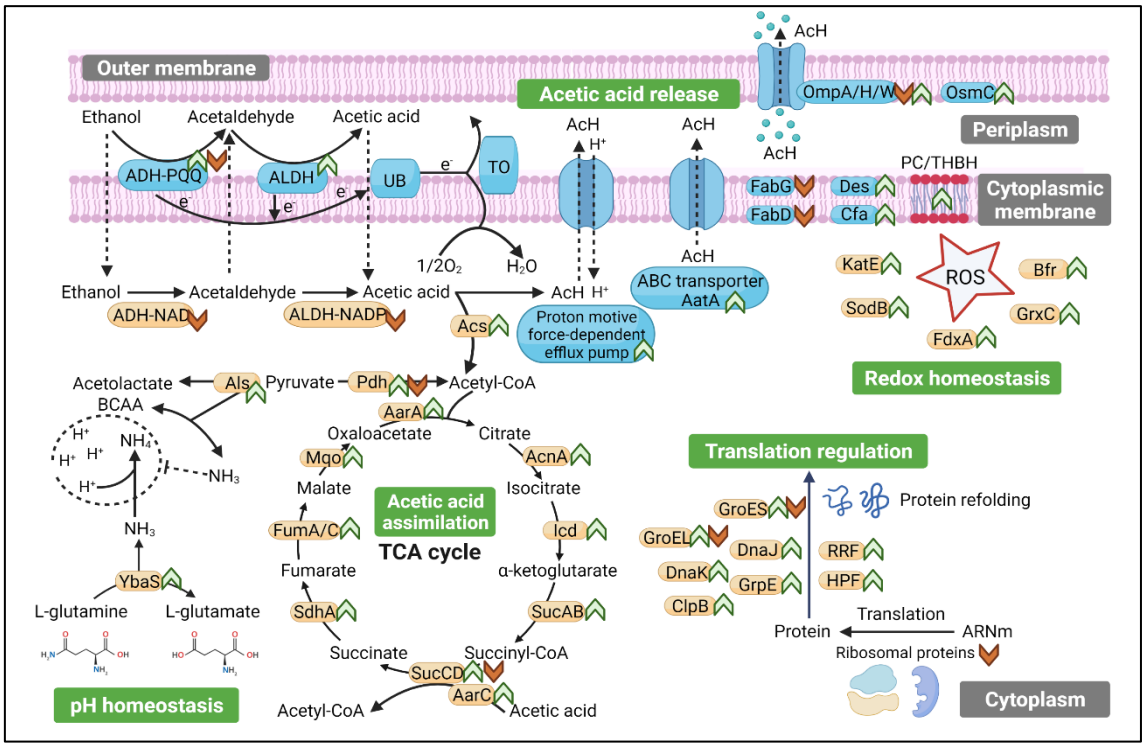

Figure 9. Main metabolic pathways performed by vinegar-producing AAB microbiota under high acetic acid concentration conditions. The molecular strategies are included both (1) at the cell membrane level (proteins in blue): ADH-PQQ, PQQ-dependent alcohol dehydrogenase; ALDH, membrane-bound aldehyde dehydrogenase; UB, ubiquinone; TO, terminal oxidase; AatA, putative ABC transporter; FabD/G, acyl-carrier transacylase; Des, fatty acid dehydrogenase; Cfa, cyclopropane fatty acid synthase; Omp, outer membrane family protein; OsmC, osmotically inducible protein C; and (2) at the cytoplasm level (proteins in orange): ADH-NAD, NAD-dependent alcohol dehydrogenase; ALDH-NADP, NADP-dependent aldehyde dehydrogenase; Acs, acetyl-CoA synthase; AarA, citrate synthase; AcnA, aconitate hydratase; Icd, NAD^+ isocitrate dehydrogenase; SucAB, α-ketoglutarate dehydrogenase; SucCD, succinyl-CoA synthetase; AarC, succinyl-CoA transferase; SdhA, succinate dehydrogenase; FumA/C, fumarate hydratase; Mqo, malate dehydrogenase; Pdh, pyruvate dehydrogenase; Als, acetolactate synthase; YbaS, glutaminase S; GroESL, heat shock proteins; DnaKJ/GrpE/ClpB, molecular chaperonin proteins; RRF, ribosome recycling factor; HPF, hibernation promoting factor; KatE, catalase; SodB, superoxide dismutase; FdxA, ferredoxin; GrxC, glutaredoxin; Bfr, bacterioferritin. Next to each protein, it is shown if is normally upregulated (green arrow) or downregulated (red arrow) based on studies to date. AcH, acetic acid; BCAA, brain-chain amino acids; NH_3, gaseous ammonia; NH_4, ammonium; PC, phosphatidylcholine; THBH, tetrahydroxybacteriohopane; ROS, reactive oxygen species. **Source**: own elaboration.

1.3.4. *Komagataeibacter europaeus*: a crucial species for industrial vinegar production

Komagataeibacter europaeus was initially observed by Sievers et al. 1992 (then named *Acetobacter europaeus*) and subsequently isolated and characterized from high-acid vinegar fermentations in central Europe. This species has been described as one of the most suitable AAB for the industrial production of vinegar because of its particular growing conditions that include high ethanol-oxidizing capability and high acetic acid-producing capability that entails both its requirement and tolerance. Consequently, they can also grow at pH 2.5 and require a constant oxygen supply (Sievers et al., 1992; Yamada et al., 2012; Gullo et al., 2014). These metabolic features allow its growth in any vinegar produced by submerged culture, especially wine vinegar [4.5-10% (w/v)] and spirit vinegar [10-20% (w/v)], but also in low-acidity vinegar [4-7% (w/v)] such as traditional balsamic vinegar and cereal vinegar (Gullo et al., 2009; Fernández-Pérez et al., 2010; Andrés-Barrao et al., 2011; Mamlouk and Gullo, 2013).

K. europaeus is normally imposed on the rest of the microbiota working in the industrial production of vinegar and currently, numerous metabolic strategies are being studied by which different strains of this species can achieve it. The main strategies used according to the literature are shown in Figure 10. Like other *Komagataeibacter* species, the respiratory chain coenzyme Q used is Q10 (Qiu et al., 2021). Moreover, PQQ-ADH is the key enzyme responsible to oxidize ethanol into acetic acid and its enzymatic activity in *K. europaeus* cells under high acidity is two times greater than that of *A. pasteurianus* (Rajpurohit et al., 2008). A higher ADH activity can result in a bigger energy pool available for membrane-associated processes such as the acetate/acetic acid export systems (Gullo et al., 2014). Integrated analysis of published AAB genomes showed differences in the number of gene copies of PQQ-ADH. The genus *Komagataeibacter* contains the most encoding gene copies and *K. europaeus* is one of the most containing species. Specifically, *K. europaeus* 5P3 contains six copies, while this gene is absent from *K. hansenii* ATCC 23769 and *K. medellinensis* NBRC 3288. The differences in the number of PQQ-ADH genes may be crucial to prevail and dominate throughout high-acid acetification processes (Wang et al., 2015b; Qiu et al., 2021).

The modification of the cellular structure and membrane composition are also important molecular strategies to consider, see Figure 10. *K. europaeus* may adapt its initial short

rod shape in absence of acetic acid to form longer and thinner rods as the acidity level increases. This morphologic change decreases the effective area for passive diffusion and storage of acetic acid into cells, enabling them to tolerate higher activity levels (Trček et al., 2007; Qiu et al, 2021). *K. europaeus* has been implied in the increase of lipid content of its cell membrane such as PC and THBH as well as the absence of CPS as differential strategies against other AAB to impose themselves throughout submerged fermentation (Trček et al., 2007; Matsushita et al., 2016; Nakano and Esibuya, 2016). Recently, an O-antigen polysaccharide fraction (Ke-PS) was isolated from *K. europaeus* NBRC 3261 and might be involved in acetic acid resistance mechanisms (Devanthéry et al., 2020). The population dynamics of submerged vinegar production using starting mixes of different AAB describes, through different metagenomics tools, that although *A. pasteurianus* is one of the predominant species at the beginning of the cycle, mainly at high ethanol content [5-7% (v/v)], *K. europaeus* always prevails after the start of the fermentation phase (Fernández-Pérez et al., 2010; Andrés-Barrao et al., 2011).

In the SSF of Chinese cereal vinegar, it has been defined that non-abundant microbiota communities play fundamental roles in network stability. Peng et al. 2021 identified *K. europaeus* as the most co-occurrent non-abundant species with an essential role in the function and resilience of the microbial community. Bioaugmentation of *K. europaeus* JNP1 verified that it may modulate the composition of the microbiota and improve the bioprocess efficiency by increasing acetic acid content and decreasing reducing sugar content (Figure 10). *K. europaeus* is capable to confer stability to the microbiota thus enhancing the final vinegar properties both in the microbiota that predominate and in those that do not.

In short, a better understanding of the molecular mechanisms used by this species to adapt to the medium and impose itself on the rest of the microbiota is necessary to improve the fermentation conditions. The characterization of suitable strains of *K. europaeus* would undoubtedly improve the features of the starter cultures currently employed for the obtention of final products with better organoleptic properties and higher quality.

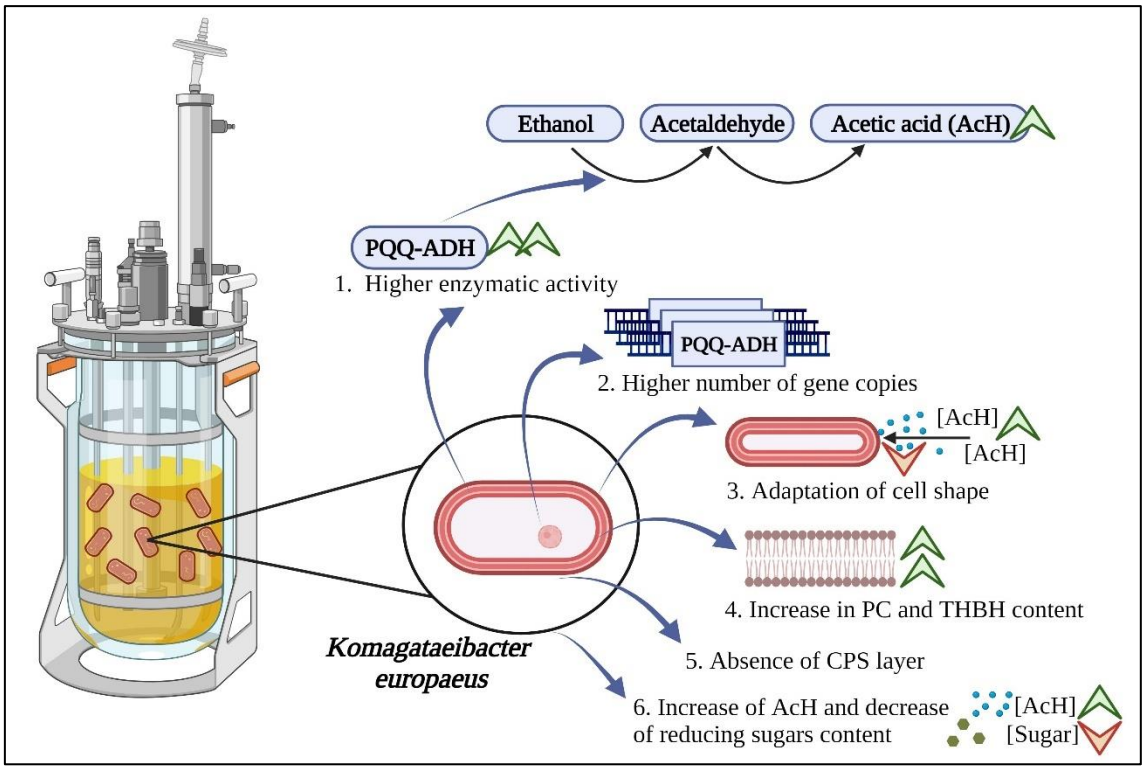

Figure 10. Different strategies used by strains of *Komagataeibacter europaeus* to prevail and impose themselves on the rest of the microbiota throughout the submerged acetification process. AcH, acetic acid; CPS, capsular polysaccharides; PC, phosphatidylcholine; PQQ-ADH, PQQ-dependent alcohol dehydrogenase; THBH, tetrahydroxybacteriohopane. **Source**: own elaboration.

1.4. Conclusions

This review has attempted to summarize the current state of knowledge on vinegar production from the diversity of raw materials and starter cultures to systems production and operating conditions used. Because of the multiple variables that influence vinegar production, it is not easy for the industries to achieve a balance between them aimed at the optimization of this process.

Considering current research and the evolution of the agri-food market, the improvement of organoleptic properties of these unique products will be focused on the implementation of new operating conditions, the characterization of new raw materials, and the study of the microbial composition and behavior of the microbiota inhabiting vinegar. “Omics sciences” emerge in the last years as one of the best tools to approach these strategies with high throughput without compromising the fitness of microbiota and the quality of the final product. The integrated study in these areas can establish the

first steps of a path toward obtaining new types of vinegar and other bioproducts with acetic acid as the main component in order to satisfy the current consumer preferences.

Section 2

HYPOTHESIS AND OBJECTIVES

2. HYPOTHESIS AND OBJECTIVES

2.1. Starting hypothesis

The elaboration of submerged vinegar is the result of a biotransformation process in which, essentially, the ethanol coming from an alcoholic raw material is converted into acetic acid. Although the technical aspects of vinegar production are currently well-known, fundamental features that finally control the activity of the microorganisms responsible for the process, complex microbiota of acetic acid bacteria, are not yet exhaustively known. Because of their particular growing conditions, acetic acid bacteria are difficult to isolate outside the bioreactors, where are fully developed thus hindering the identification and understanding of their molecular mechanisms. Strategies focused on the imposition and survival of the predominant microbiota, as well as the adaptation of the minor population making a stable community are of special interest. "Omics sciences" allow for the massive analysis of macromolecules and metabolites coming from these microorganisms working in their natural media to offer a precise picture of what is occurring throughout acetification. Metagenomics, metatranscriptomics, metaproteomics, and metabolomics applied to "shotgun" technology consist of innovative approaches that allow determining genes, proteins, and metabolites content, respectively, of complex microbiota. Presently, these methodologies are starting to be implemented in the vinegar industry field. The updating of the existing knowledge of interactions between substrate, microbiota, and multi-omics would allow for improving the quality of vinegars and the development of new ones with sensory and bio-healthy profiles adapted to the Andalusian agri-food sector.

In light of the above-mentioned, the proposed starting hypothesis aims to approach three premises. First, there are possible differences in the microbial diversity and function of the vinegar-producing microbiota throughout submerged acetification. Second, the use of different raw materials may influence the characterization of these acetification profiles. Third, these findings may be verified and compared under different omics approaches.

2.2. Objectives

2.2.1. General objective

Because of the foregoing, the following general objective has been proposed:

To characterize and compare the development of three acetification profiles to study the evolution of operating conditions and the effect of diverse raw materials used (synthetic alcohol-based medium, fine wine, and craft beer) on the composition and the behavior of the microbiota responsible for the process.

2.2.2. Specific objectives

1) To characterize and compare the metaproteome, qualitatively and quantitatively, of the microbiota responsible for three acetification profiles (synthetic alcohol-based medium, fine wine, and craft beer) and evaluate the differential variables throughout the process.

This objective has been approached from three aspects:

I. To study the variations of system variables, as well as of the composition and main functions of the microbiota present throughout the evolution of an acetification process using a reference raw material (synthetic alcohol-based medium) through a qualitative metaproteomic approach.

II. To explore the quantitative differences of the metaproteome, especially on the particular proteome of the predominant microbiota, and its interactions with the non-abundant community throughout the acetification profile of synthetic alcohol-based medium.

III. To characterize two acetification processes from natural raw materials (fine wine and craft beer) and compare the influence of each one on the metaproteome of both the predominant and minor microbiota, especially, through the molecular strategies used for adaptation and survival.

2) To confirm the composition of the microbiota obtained through metaproteomics by applying metagenomics tools, as well as the collection and characterization of isolates from the samples taken throughout the acetification of the three working media: synthetic alcohol-based medium, fine wine, and craft beer.

3) To characterize the "volatilome" and differentiate the key volatile compounds throughout the evolution of the acetification of the three raw materials: synthetic alcohol-based medium, fine wine, and craft beer.

2.2.3. Publications covering each objective

1) Publications for specific Objective 1

I. Román-Camacho, J. J., Santos-Dueñas, I. M., García-García, I., Moreno-García, J., García-Martínez, T., Mauricio, J. C. (2020). Metaproteomics of microbiota involved in submerged culture production of alcohol wine vinegar: A first approach. *International Journal of Food Microbiology*. 333, 108797. https://doi.org/10.1016/j.ijfoodmicro.2020.108797.

II. Román-Camacho, J. J., Mauricio, J. C., Santos-Dueñas, I. M., García-Martínez, T., García-García, I. (2021). Functional metaproteomic analysis of alcohol vinegar microbiota during an acetification process: A quantitative proteomic approach. *Food Microbiology*. 98, 103799. https://doi.org/10.1016/j.fm.2021.103799.

III. Román-Camacho, J. J., Mauricio, J. C., Santos-Dueñas, I. M., García-Martínez, T., García-García, I. (2022). Unraveling the role of acetic acid bacteria comparing two acetification profiles from natural raw materials: a quantitative approach in *Komagataeibacter europaeus*. *Frontiers in Microbiology*. 13, 840119. https://doi.org/10.3389/fmicb.2022.840119.

2) Publications for specific Objective 2

IV. Román-Camacho, J. J., García-García, I., Santos-Dueñas, I. M., Ehrenreich, A., Liebl, W., García-Martínez, T., Mauricio, J. C. (2022). Combining omics tools for the characterization of the microbiota of diverse vinegars obtained by submerged culture: 16S rRNA Amplicon Sequencing and MALDI-TOF MS. *Frontiers in Microbiology* (**ACCEPTED**).

3) Publications for specific Objective 3

The results obtained from this specific objective are shown, at this time, as unpublished results.

Section 3

RESULTS AND DISCUSSION

3. RESULTS AND DISCUSSION

The results obtained throughout this work are presented below in different sections according to each one of the specific objectives proposed (see section 2.2.2.).

3.1. Metaproteomics of the microbiota responsible for the acetification of three raw materials: synthetic alcohol-based medium, fine wine, and craft beer

Several fundamental aspects of the molecular mechanisms driving the overall activity of complex microbiota of acetic acid bacteria (AAB) are not yet comprehensively well-known despite their importance in vinegar production. Metaproteomics provides an innovative approach to studying microbial communities inhabiting vinegar without the requirement to isolate them outside their natural media, which entails serious difficulties to perform. Moreover, it allows for the massive analysis of proteins providing wide and precise information about the composition and behavior of the microbiota throughout acetification. In this section, the results obtained through metaproteomic studies, both at a qualitative and quantitative level, of the microbiota responsible for three submerged acetification processes by using first, a reference raw material (synthetic alcohol-based medium) and second, two natural raw materials (fine wine and craft beer) are presented. This research is approached from three works that, as a whole, allow for the characterization and comparison of three acetification profiles in order to evaluate the differential variables throughout the process.

3.1.1. Chapter I. Metaproteomics of the microbiota throughout the evolution of the acetification of synthetic alcohol-based medium. Study of the microbial composition and functions through a qualitative approach

In this first work, vinegar from a synthetic alcohol-based medium was produced through a submerged culture of acetic acid bacteria using a pilot acetator, operated in a semi-continuous mode, where the main system variables were monitored. Metaproteomic analysis was performed at crucial moments of the acetification cycle (at the end of fast and discontinuous loading phases and just before the unloading stage) by the use of liquid chromatography with mass tandem spectrometry (LC-MS/MS) technology. Through a first qualitative approach, it is aimed to evaluate the system variables, as well as characterize the composition and behavior of the existing microbiota and its possible variations throughout the course of the acetification process.

The results from the synthetic alcohol-based medium acetification profiling supported that the main system variables induced variations in the mean values of some properties of the culture medium, mainly determined by the semi-continuous state of the cycles leading to ethanol concentration sudden changes and increasing levels of acetic acid, which may directly influence the behavior and stress response of AAB microbiota. Metaproteomic analysis revealed that the microbiota was composed, in terms of protein abundance, mainly of *Komagataeibacter* (85.66%), *Acetobacter* (5.34%), *Gluconacetobacter* (1.63%), and *Gluconobacter* (1.10%) contributing these four genera with 1,675 out of 1,723 (93.73%) total proteins belonging up to 30 different genera of the Acetobacteraceae family. The species *Komagataeibacter europaeus* provided the highest protein amount (73.67% of total proteins), far above the rest ones. It is worth noting that no relevant differences in the number of proteins throughout the acetification cycle were found, probably because of the high number (49.39%) of common proteins at the sampling times. GO Term enrichment analysis highlighted the important role of the catalytic activity, organic cyclic compound binding, and metabolic processes of biosynthesis throughout acetic acid fermentation. These results proposed a microbiota composition in which *K. europaeus* is the predominant species and a minor fraction of less-abundant species, including both species closely related to *K. europaeus*, and others never before described in vinegar to date, might coexist and contribute to the whole microbiota role. The well-known particular growing conditions of *K. europaeus* endorse its suitability to prevail over the rest of the vinegar microbiota.

This first qualitative metaproteomic study allowed to get an overview of the biodiversity and behavior of the microorganisms which participate in alcohol-based vinegar making and establish the basis of a reference acetification profile to compare with subsequent works using both different alcoholic raw materials and operating conditions. These findings might contribute to the optimization of the methodology and the improvement of the quality of the final products. Despite this, exhaustive protein quantification and differential expression analyses will be essential for a better understanding of the molecular strategies used by the AAB microbiota throughout the submerged acetification (see section 3.1.2.).

The results which comprise this work have been published in the journal *International Journal of Food Microbiology* with the title "*Metaproteomics of microbiota involved in submerged culture production of alcohol wine vinegar: A first approach*", see section 6.1.1., appendix.

International Journal of Food Microbiology 333 (2020) 108797; https://doi.org/10.1016/j.ijfoodmicro.2020.108797.

3.1.2. Chapter II. Quantitative metaproteomics for the characterization of alcohol-based vinegar microbiota. A comprehensive functional analysis of the *Komagataeibacter europaeus* proteome

In this work, as a continuation of the first one (section 3.1.1.), the characterization of the reference acetification profile of synthetic alcohol-based medium was completed at the technical and microbiological levels. A "shotgun" metaproteomic strategy was carried out consisting of the identification and quantification of the metaproteome through LC-MS/MS and subsequent exhaustive bioinformatic analysis of the proteome of the most predominant species in terms of protein amount, *K. europaeus*. The detection of differential quantification changes of the main species may contribute to predicting the metaproteome function.

A total of 78 species of 25 different genera were found from 1,361 proteins identified in the alcohol-based vinegar metaproteome. *Komagataeibacter* species provided more than 90% of the total proteins highlighting *K. europaeus*, which accounts for around 74%. Metaproteome function analysis of the main *Komagataeibacter* species and, briefly, of the minor species, described the natural behavior of the AAB microbiota. Non-abundant species might survive and establish a stable coexistence and functional relationship with the predominant species, *K. europaeus*, which manifested a key role in the function of the vinegar microbiota. For these reasons, the proteome of *K. europaeus* was subjected to enrichment LC-MS/MS and detailed bioinformatic analyses. These results confirmed the importance of some metabolic processes of macromolecules, such as amino acids (biosynthesis and deamination) and proteins (aminoacylation of tRNAs, formation of ribosomes, and translation), as well as energy (ATP) generation related pathways (TCA cycle, pentose phosphate pathway, and glycolysis), whose proteins increased their quantification values throughout the loading phase. Afterward, these proteins suffered a quantification decrease and came into play other proteins involved in the acetic acid resistance at the final moments of the acetification, just before unloading.

This work, as a continuation of the previous one, could be a further step towards achieving a better understanding of the role of the vinegar microbiota, especially by focusing on the predominant acetic acid bacteria and their interactions with the less-abundant population. The characterization of the alcohol-based medium may establish a

reference acetification profile, because of its synthetic origin, for comparison with further works using different natural raw materials (section 3.1.3).

The results obtained in this work have been published in the journal *Food Microbiology* with the title "*Functional metaproteomic analysis of alcohol vinegar microbiota during an acetification process: A quantitative proteomic approach*", see section 6.1.1., appendix.

Food Microbiology 98 (2021) 103799; https://doi.org/10.1016/j.fm.2021.103799.

3.1.3. Chapter III. Characterization and comparison of two acetification profiles using natural raw materials (fine wine and craft beer) and their influence on the quantitative proteome of *Komagataeibacter europaeus*

In this work, two submerged acetification profiles coming from the same starter inoculum of acetic acid bacteria but using two natural raw materials of different origins (fine wine and craft beer), were characterized and compared mainly focusing on the study of the effect of these raw materials. It is worth noting that the starter inoculum consisted of a mixed culture coming from our previous works, concretely, harvested at the final of a fully active acetification process making alcohol-based vinegar (sections 3.1.1. and 3.1.2.). The composition and natural behavior of AAB microbiota inhabiting vinegar throughout the acetification of both raw materials were compared through "shotgun" metaproteomics using LC-MS/MS, especially focusing on the protein profile of *K. europaeus* from a quantitative approach. This species was selected, as in our previous studies, because it provided the highest amount of the metaproteome (73.5%) and played an essential role in the microbial community function.

The results of the characterization of the two acetification processes showed significant differences as a function of the available nutrients. The remarkable presence of sugars in the craft beer medium might lead to the activation of several metabolic pathways aimed at taking advantage of this nutrient source. Similarly, fine wine not only allowed higher acetification rates but also higher final acidity levels, thus making harsher environmental conditions that could lead to the activation of other metabolic strategies in response to stress. A total of 1,069 and 1,268 proteins were identified in the LC/MS-MS analysis in fine wine and craft beer media, respectively. Although proteins from 84 different species of AAB were found, only 13 of them constituted around 90% of the metaproteome (11 species from *Komagataeibacter* as well as *Acetobacter* sp. and *Gluconacetobacter* sp.). *K. europaeus* was the most abundant species providing a mean protein frequency of 73.5%. It is interesting to note that despite working with two different raw materials, no significant changes regarding the microbial composition of the protein profiles were observed but they influenced the protein abundance. A later enrichment and quantitative proteomic analysis of *K. europaeus* revealed significant changes depending on the raw material and sampling phase. A molecular strategy in which *K. europaeus* might prevail over the rest of the microbiota by taking advantage of the nutritional features of each raw material based on three aspects was proposed: (1) by

metabolizing the excess of cytoplasmic acetic acid using the TCA cycle and supplying biosynthetic precursors to replenish the cellular material losses; (2) by previous use of the excess of available glucose, mainly in the craft beer medium, through the pentose phosphate pathway and the glycolysis; (3) by triggering membrane mechanisms proton motive force-dependent to detoxify the cell at the end of the acetification.

The characterization of the acetification of these two natural raw materials, with high nutritional richness, may lay the groundwork for the use of new raw materials as acetification substrates to propose new types of vinegar. Although these metaproteomic approaches, particularly focused on the quantitative protein profile of the predominant microbiota, have allowed obtaining a better knowledge about the microbial composition and the molecular strategies used by these microorganisms throughout submerged acetification, the confirmation of these findings using other omics tools should be considered (section 3.2.). Additional metabolomic assays may be necessary to determine the metabolite profiles from the raw materials to the final products, as well as clarify their main differences (section 3.3.).

These findings have been published in the journal *Frontiers in Microbiology* with the title "*Unraveling the role of acetic acid bacteria comparing two acetification profiles from natural raw materials: a quantitative approach in Komagataeibacter europaeus*", see section 6.1.1., appendix.

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Unraveling the Role of Acetic Acid Bacteria Comparing Two Acetification Profiles From Natural Raw Materials: A Quantitative Approach in *Komagataeibacter europaeus*

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The industrial production of vinegar is carried out by the activity of a complex microbiota of acetic acid bacteria (AAB) working, mainly, within bioreactors providing a quite specific and hard environment. The "omics" sciences can facilitate the identification and characterization analyses of these microbial communities, most of which are difficult to cultivate by traditional methods, outside their natural medium. In this work, two acetification profiles coming from the same AAB starter culture but using two natural raw materials of different alcoholic origins (fine wine and craft beer), were characterized and compared and the emphasis of this study is the effect of these raw materials. For this purpose, the composition and natural behavior of the microbiota present throughout these profiles were analyzed by metaproteomics focusing, mainly, on the quantitative protein profile of *Komagataeibacter europaeus*. This species provided a protein fraction significantly higher (73.5%) than the others. A submerged culture system and semi-continuous operating mode were employed for the acetification profiles and liquid chromatography with tandem mass spectrometry (LC-MS/MS) for the protein analyses. The results showed that neither of two raw materials barely modified the microbiota composition of the profiles, however, they had an effect on the protein expression changes in different biological process. A molecular strategy in which *K. europaeus* would prevail over other species by taking advantage of the different features offered by each raw material has been suggested. First, by assimilating the excess of inner acetic acid through the TCA cycle and supplying biosynthetic precursors to replenish the cellular material losses; second, by a previous assimilation of the excess of available glucose, mainly in the beer medium, through the glycolysis and the pentose

phosphate pathway (PPP); and third, by triggering membrane mechanisms dependent on proton motive force to detoxify the cell at the final moments of acetification. This study could complement the current knowledge of these bacteria as well as to expand the use of diverse raw materials and optimize operating conditions to obtain quality vinegars.

Clinical Trial Registration: [www.ClinicalTrials.gov], identifier [PXD031147].

Keywords: ***Komagataeibacter europaeus*, vinegar, fine wine, craft beer, proteomics, submerged culture**

INTRODUCTION

The industrial elaboration of vinegar is carried out through an acetification process from an alcoholic raw material obtaining a final product with high acetic acid content. The incomplete oxidation of the ethanol into acetic acid is performed by acetic acid bacteria (AAB), strictly aerobic microorganisms that, among their several biotechnological applications, are primarily responsible for this process of biotransformation that occurs within industrial reactors (García-García et al., 2007; Mamlouk and Gullo, 2013).

The quality of the vinegar depends on many factors including the microbial composition, the raw material, and operating conditions (Mas et al., 2014; Li et al., 2015). Regarding microbial composition, several studies have demonstrated that vinegar is a product resulting from the metabolism of a complex AAB microbiota, not by pure species (Trček et al., 2016; Román-Camacho et al., 2020). This microbiota is mostly composed of species from the genera *Acetobacter* and *Komagataeibacter* (many of them relocated from *Gluconacetobacter*) which are imposed because of their high capabilities for vinegar production, although species from other genera might coexist with the best-adapted ones (Gullo et al., 2014; Wang et al., 2015). The raw material employed as acetification substrate plays an essential role in the quality of the final product. High-quality wines allow to elaborate some of the most appreciated vinegars in the world, however, other alcoholic substrates including cereals (rice, malt, wheat, corn, and among others), fruits, and apple cider are also well-known (Hidalgo et al., 2013; Trček et al., 2016; Zhang et al., 2019; Kandylis et al., 2021; Peng et al., 2021). Conversely, vinegar is mainly produced at the industrial scale by submerged cultures in reactors that continuously supply very fine air bubbles into the medium as an aeration mechanism. The submerged system has several advantages over other techniques, such as solid-state fermentation or surface fermentation including high yield and process speed (Gullo et al., 2014). Through a semi-continuous operating mode, in which each cycle starts by loading the tank with fresh medium to a preset volume and finishes when a part of the volume is unloaded after depleting ethanol to an also preset concentration, high productivity and stability are ensured (Jiménez-Hornero et al., 2020). This working mode allows part of the biomass produced in each cycle to rapidly start the next one. Also, the operational variables can be used to maintain the average substrate and product concentrations within appropriate ranges for AAB to operate, which in turn, facilitates self-selection and adjustment to the specific medium (García-García et al., 2019).

The particular growing conditions and metabolic characteristics of AAB hinder their isolation outside the environments in which they carry out their activity fully (Fernández-Pérez et al., 2010; Mamlouk and Gullo, 2013). This fact limits the study of the richness and biodiversity of these microbiota that inhabit aggressive media as is the case of vinegar. The "omics" sciences can facilitate the analysis of the identification and function of complex microbiomes and resolve the hurdles of traditional methods for the characterization of either non-cultivable or hard to cultivate microorganisms (Andrés-Barrao et al., 2016; Xia et al., 2016; Zhu et al., 2018; Jiang et al., 2019; Verce et al., 2019). Recently, the microbiota of an acetification process using an alcohol medium as a reference has been characterized at a metaproteomic level (Román-Camacho et al., 2020, 2021). The *Komagataeibacter* species were predominant throughout the process and *K. europaeus* provided the major fraction of proteins, far above the others. This species has been described as one of the most suitable AAB for the industrial production of vinegar because of its growing conditions that include high ethanol-oxidizing ability, acetic acid requirement, and tolerance to both low [7–9% (w/v)] and high acidity levels [10–20% (w/v)] (Trček et al., 2007; Yamada et al., 2012; Gullo et al., 2014; Peng et al., 2021).

The present work aims to characterize and compare two acetification profiles using the same starter culture, coming from an acetification of previous works (Román-Camacho et al., 2020, 2021) making alcohol vinegar, but using different raw materials. For this purpose, the composition and natural behavior of the microbiota present throughout both processes were compared employing a metaproteomic analysis and especially, focusing on the protein profile of *K. europaeus* from an exhaustive quantitative approach. This species has been selected, as in our previous studies, because it provides a considerable amount (73.5%) of the metaproteome and plays an essential role in the microbial community function. A comparison of vinegar profiles using two natural raw materials (fine wine and craft beer), with a higher nutritional richness than the reference synthetic alcohol medium (Román-Camacho et al., 2020, 2021), under a strategy that employs a submerged culture and a semi-continuous operating mode, could elucidate the effect of the raw materials

on the organoleptic properties and quality of industrially elaborated vinegars.

MATERIALS AND METHODS

Raw Material

Two different alcoholic substrates were used as fermentation media: a dry fine wine from the Montilla-Moriles region (Bodegas Alvear S.A., Montilla, Córdoba, Spain) and a craft beer (Mahou-San Miguel, Córdoba, Spain). The dry fine wine contained an initial ethanol concentration of 15% (v/v) and an amino acids content of 0.72 ± 0.20 mM for L-proline, 0.24 ± 0.03 mM for L-aspartic acid, 0.23 ± 0.21 mM for ammonium ion, 0.21 ± 0.00 mM for L-γ-aminobutyric acid, 0.19 ± 0.02 mM for L-glutamic acid, 0.16 ± 0.01 mM for L-lysine, 0.15 ± 0.01 mM for L-arginine, 0.11 ± 0.01 mM for L-tyrosine, 0.06 ± 0.01 mM for L-leucine, 0.05 ± 0.01 mM for L-valine, 0.04 ± 0.01 mM for L-histamine, 0.03 ± 0.01 mM for L-glycine, 0.02 ± 0.01 mM for L-threonine, and 0.01 ± 0.01 mM for L-tryptophan. Conversely, the craft beer was obtained from a medium containing 35% of total sugars, remaining without fermenting 7% and composed, roughly, half, and half between maple syrup and muscovado sugar. The ethanol content was 17% (v/v) and the amino acids content of 3.66 ± 0.05 mM for L-γ-aminobutyric acid, 1.44 ± 0.03 mM for L-aspartic acid, 1.21 ± 0.02 mM for L-glutamic acid, 1.05 ± 0.02 mM for L-arginine, 0.92 ± 0.02 mM for ammonium ion, 0.90 ± 0.13 mM for L-proline, 0.55 ± 0.01 mM for L-glutamine, 0.47 ± 0.01 mM for L-glycine, 0.39 ± 0.01 mM for L-phenylalanine, 0.30 ± 0.01 mM for L-tryptophan, 0.18 ± 0.01 mM for L-leucine, 0.17 ± 0.01 mM for L-tyrosine, 0.13 ± 0.01 mM for L-histidine, 0.11 ± 0.03 mM for L-threonine, 0.04 ± 0.01 mM for L-histamine, and 0.02 ± 0.01 mM for L-lysine. Both raw materials were diluted with distilled water to adjust the ethanol concentration to the working conditions [≈ 10% (v/v)] reaching 9.8 ± 0.3 and 9.5 ± 0.3% (v/v) for fine wine and beer, respectively; the initial acetic acid concentration was of 0.2 ± 0.1% (w/v).

Microorganism

The starter culture consisted of a mixed broth coming from a fully active acetification process making alcohol vinegar, concretely harvested from the end of the ethanol exhausting phase, see microbial composition in **Supplementary File 1** (Román-Camacho et al., 2020). This original alcohol medium was composed of 10% ethanol, glucose (1 g/L), calcium pantothenate (13 mg/L), calcium citrate (0.1 g/L), potassium citrate (0.1 g/L), diammonium phosphate (0.5 g/L), magnesium sulfate (0.1 g/L), manganese sulfate (5 mg/L), and iron chloride (1 mg/L) following the method of Llaguno (1991) with yeast extract (0.25 g/L) and peptone (0.5 g/L) additionally supplied. A previous stage using each specific raw material, including several cycles of acetification, is necessary to adapt the inoculum and achieve a repetitive system behavior.

Operating Mode

Acetification cycles were carried out in a fully automated 8 L Frings bioreactor (Heinrich Frings GmbH & Co., KG, Bonn, Germany) working in a semi-continuous operating mode. Each cycle is started by a loading phase that replenishes the reactor with a fresh medium to the working volume (8 L) without exceeding a preset ethanol concentration of 5% (v/v). Then, an exhausting stage occurs depleting ethanol in the culture broth to a preset concentration of 1.0–1.5% (v/v). Finally, 50% of the volume is fast unloaded and the remaining content is used as inoculum of the next cycle. A constant temperature of 31°C, a fast-loading rate of 1.3 L/h, and an air-flow rate of 7.5 L/(h L medium) were employed. Sigmaplot 12.0 (Systat Software Inc., CA, United States) was used for graphical representation of the acetification profiles after monitoring the system data by LabView application (National Instruments, TX, United States). **Figure 1** shows the profiles of the main variables.

Sampling

Sampling was performed at two relevant times of the acetification cycle: at the end of the loading phase (EL), when final working volume or preset ethanol concentration of 5% (v/v) is reached, whichever occurs first; and just before the unloading phase, at the end of the ethanol exhaustion (UL). A total of 15 acetification cycles for each vinegar profile were performed including some previous cycles (7–10) necessary to achieve a semi-continuous repetitive state of the system. Six samples were harvested from fine wine vinegar: three at EL (cycles 12, 14, and 15) and three at UL (cycles 11, 13, and 14); and seven samples from beer vinegar: four at EL (cycles 11, 12, 13, and 14) and three at UL (cycles 12, 13, and 14).

Analytical Methods

System variables including the volume of the medium (L), ethanol concentration % (v/v), and temperature (°C) were constantly measured using an EJA 110 differential pressure probe (Yokogawa Electric Corporation, Tokyo, Japan), an Alkosens® probe (Heinrich Frings GmbH & Co., KG, Bonn, Germany), and a temperature probe, respectively. The automatization of the system allows the continuous recording of data as well as testing the high reproducibility of the method. Acetic acid concentration %, (w/v) was determined by acid-base titration with 0.5 N NaOH. Viable cells concentration, the difference between total and no viable cells, were directly counted using a light microscope (Olympus BX51), a Neubauer chamber (Blaubrand™, 7178-10) with 0.02 mm depth and rhodium-coated bottom, and propidium iodide (VWR, Inc., PA, United States). Though the chamber was subdivided into 25 square groups, composed of 16 squares each, 5 square groups (0.04 mm^2 each) on the diagonal were used for cell counting following the method of Baena-Ruano et al. (2006); samples were quantified by triplicate and standard error was calculated. These variables were exclusively measured at sampling times. The efficiency of the process was evaluated by mean acetification rate (r_A) and

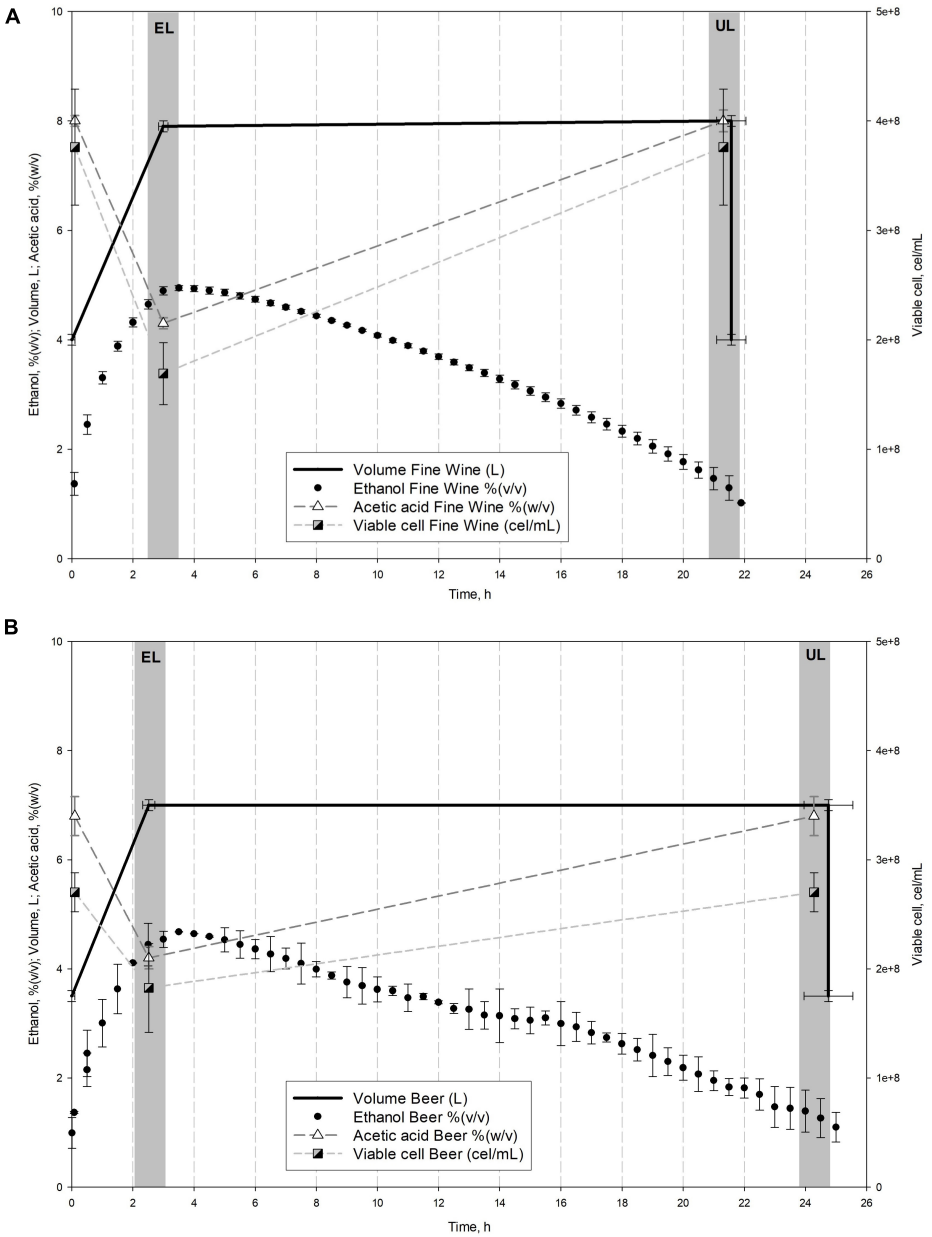

FIGURE 1 | Comparison of the main system variables throughout fine wine **(A)** and beer **(B)** vinegar profiles. Mean values and standard deviation (*SD*) of the variables of stable cycles performed are represented. Sampling times (EL, end of loading; UL, just before unloading) are also shown.

global production of acetic acid (p_A) which were calculated as follows:

$$r_A = \frac{\begin{array}{c}Final\ acetic\ acid\ concentration\ (\%,\ w/v)\\ \times Unloaded\ volume\ (L)\end{array}}{Total\ cycle\ time\ (h)\ \times\ Mean\ cycle\ volume\ (L)}$$

$$p_A = \frac{\begin{array}{c}Final\ acetic\ acid\ concentration\ (\%,\ w/v)\\ \times\ Unloaded\ volume\ (L)\end{array}}{Total\ cycle\ time\ (h)}$$

Proteomics

Sample Processing

Vinegar samples were harvested by directly unloading a volume of 300 mL from the pilot acetator, dividing it into six fractions of 50 mL each, and putting them in centrifuge tubes on ice. Cells were separated by centrifugation and then, twice cleaned using cold sterile distilled water; the resulting pellets were stored at −80°C. Then, cell extracts were broken by several cycles using glass beads and sonication after adding extraction buffer (100 mM Tris-HCl buffer pH 8.0, 2 mM dithiothreitol (DTT), 1 mM ethylenediaminetetraacetic acid (EDTA), and

1 mM phenylmethylsulphonyl fluoride (PMSF) supplemented with Protease Inhibitor Cocktail tablets). The protein fraction was precipitated, vacuum dried, solubilized, and its concentration was quantified by Bradford (1976) assays. A volume of each protein sample containing 50 μg was injected into LC-MS/MS analysis at Research Support Central Service (SCAI), University of Córdoba, Spain. All proteomic procedures were performed following the methodology previously developed by our group (Román-Camacho et al., 2020).

Protein Identification by Database Searching

Mass spectrometry raw data were processed using Proteome Discoverer (version 2.1.0.81, Thermo Fisher Scientific, MA, United States). MS/MS spectra were searched with SEQUEST engine against Uniprot.[1] Peptides obtained from tryptic digestion were searched setting the following parameters: up to one missed cleavage, cysteine carbamidomethylation as a fixed modification, and methionine oxidation as a variable one. Precursor mass tolerance was 10 ppm while ion products were searched at 0.1 Da tolerances. Peptide spectral matches (PSM) validation was performed at a 1% FDR using a percolator based on *q*-values. Peptide quantification was carried out by calculating precursor ion areas by Precursor Ion Area Detector and normalizing by Total Peptide Amount mode of Proteome Discoverer. The parsimony law was applied to obtain protein groups and filtered to 1% FDR.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [1] partner repository with the dataset identifier PXD031147.

Raw Data Analysis

Proteins identified in the metaproteome were first screened removing those with score < 2 and number of peptides ≤ 2. Then, proteins in at least 50% out of total samples (three or four) in at least one sampling time were maintained. Exclusive proteins were obtained by the difference between those aforementioned and those identified in at least 50% out of total samples in each sampling time. From those, a GO Term analysis using Uniprot and Gene Ontology (GO) annotation tool[2] was performed to detail the metaproteome function. Subsequently, an enrichment analysis LC-MS[2] of the proteome of *K. europaeus* was performed and quantitative changes throughout each acetification profile were compared. Protein values were normalized by dividing each one by the sample global intensity and then multiplied by the mean value of global intensity from all samples. First, those proteins obtained in at least 50% of samples in one sampling time were retained and plotted in an intersection diagram ("UpSetR" R library). For the hierarchical clustering and heat map analysis, proteins identified in at least 50% of samples in each sampling time were used. Mean quantification values were previously scaled, centered by z-score transformation, and then, Pearson correlation was applied with method "complete" ("hclust" function in stats package from R). One-Way ANOVA followed by HSD Tukey's test was calculated by R functions "lm" and "anova" and *q*-value was used to calculate *p*-value multiple testing correction. Proteins identified only in one biological replicate were eliminated from the overall count.

Furthermore, the biological function of the protein clusters was studied by building protein-protein interaction network maps (INM) by using STRING database v11.[3] High confidence interaction (score = 0.70–0.90) and protein annotations based on the databases Uniprot (see text footnote 1) and KEGG[4] were used (see **Supplementary File 2**). Because *K. europaeus* is not available in the database, as in previous works (Román-Camacho et al., 2021), *K. xylinus* E25, a closely related species (MUM index of 0.21, according to Ryngajłło et al., 2018), was used as a model organism due to the high genome homology.

[1]http://www.uniprot.org

[2]http://geneontology.org/

RESULTS

Description of Fine Wine and Beer Acetification Profiles: A Comparison

Figure 1 shows a comparison of the mean cycle of main system variables throughout fine wine (**Figure 1A**) and beer (**Figure 1B**) profiles, while **Table 1** lists the mean values of the aforementioned variables. The fine wine profile showed a fast-loading phase up to reach the working volume (8.0 ± 0.1 L) and an ethanol concentration of 4.9 ± 0.0% (v/v) at the end of the stage, at 3.0 ± 0.0 h. An exhausting phase started with the depletion of ethanol content up to 1.3 ± 0.3% (v/v) and just then, 50% of the reactor volume was unloaded (4.0 ± 0.1 L), at 21.4 ± 0.1 h. During this period, both acetic acid concentration [from 4.3 ± 0.0 to 7.9 ± 0.2% (w/v)] and cell viable concentration (from 1.43 ± 0.33 to 1.47 ± 0.28 × 10^8 cel/mL) were increased. The beer profile was operated with a final working volume of 7.0 ± 0.2 L because of the excessive foaming. First, a continuous

[3]https://string-db.org/

[4]https://www.genome.jp/kegg/

TABLE 1 | Main variables of the acetification profile including both the system variables constantly monitored and those exclusively measured at sampling times.

	Variable	FW_EL	FW_UL	B_EL	B_UL
Mean ± *SD*	Cycle time (h)	3.0 ± 0.0	21.4 ± 0.1	2.8 ± 0.4	24.3 ± 1.1
	Volume (L)	8.0 ± 0.1	8.0 ± 0.1	7.0 ± 0.2	7.0 ± 0.2
	Ethanol (% v/v)	4.9 ± 0.0	1.3 ± 0.3	4.7 ± 0.2	1.2 ± 0.1
	Acetic acid (% w/v)	4.3 ± 0.0	7.9 ± 0.2	4.2 ± 0.4	6.8 ± 0.7
	Viable cell (10^8 cel/mL)	1.43 ± 0.33	1.47 ± 0.28	0.84 ± 0.70	1.05 ± 0.70
		FW		B	
	Mean acetification rate (r_A) [g acetic acid/(L h)]	0.19 ± 0.01		0.16 ± 0.01	
	Global acetic acid production (p_A) (g acetic acid/h)	15.2 ± 0.5		11.3 ± 0.5	

Data show mean values of all variables at the sampling times (FW_EL, FW_UL, B_EL, B_UL) and their standard deviation (SD). Variables used to obtain the acetification efficiency of each profile are included.

fast loading was performed to the aforementioned volume and an ethanol concentration of 4.7 ± 0.2% (v/v), both achieved at 2.8 ± 0.4 h. The second phase (24.3 ± 1.1 h) concluded when ethanol concentration was depleted to 1.2 ± 0.1% (v/v) and 50% of the volume of the medium was unloaded (3.5 ± 0.1 L). At the same time, the acetic acid concentration [from 4.2 ± 0.4 to 6.8 ± 0.7% (w/v)] and cell viability (from 0.84 ± 0.70 to 1.05 ± 0.70 × 10^8 cel/mL) were increased throughout this exhausting period. The efficiency of each acetification profile was evaluated by the mean acetification rate (r_A) and global production of acetic acid (p_A), both calculated as described in section "Analytical Methods" (see **Table 1**).

It is interesting to note that the two raw materials used show some significant differences to evaluate the behavior of the microbiota as a function of the available nutrients. In particular, the significant presence of sugars in the craft beer medium could lead, as it will be discussed later in this work, to the activation of several metabolic pathways aimed at taking advantage of this resource. Similarly, fine wine, a substrate whose suitability as an acetification medium is well known, not only allows higher acetification rates, but also higher final acidity values, which leads to harsher environmental conditions. This fact can trigger the activation of metabolic pathways other than those mentioned above in response to stress.

Comparison of the Metaproteome of Fine Wine and Beer Vinegar

Microbial Composition

A total of 1,069 (EL, 934; UL, 945) and 1,268 (EL, 1,226; UL, 1,110) proteins were identified in the LC/MS-MS analysis in fine wine and beer vinegar samples, respectively, after removing contaminants and those proteins not found in at least 50% of the samples in at least one sampling time (see **Supplementary File 1**). Although proteins belonging to 84 different species from the *Acetobacteraceae* family were identified, only 13 of them constituted around 90% of the metaproteome (see **Supplementary Table 1**): 11 species from the *Komagataeibacter* genus (*K. europaeus*, *K. xylinus*, *K. intermedius*, *K. rhaeticus*, *K. diospyri*, *K. swingsii*, *K. medellinensis*, *K. nataicola*, *K. oboediens*, *K.* sp., and *K. sucrofermentans*) as well as *Acetobacter* sp. and *Gluconacetobacter* sp.; *K. europaeus* was the most abundant species providing the largest amount of proteins (73.5%: FW_EL, 75.9%; FW_UL, 75.0%; B_EL, 70.6%; B_UL, 72.6%), far above the rest of species. No relevant differences regarding the composition of the microbiota were observed between sampling times and profiles. Since none of the remaining species exceeds a mean frequency of 0.5%, the functional metaproteome analysis is mainly focused on this major amount, which is considered sufficiently representative of the total (Román-Camacho et al., 2021).

Gene Ontology Term Functional Analysis

Because a high amount of the metaproteome (834 proteins) was common when different raw materials were used during the acetification, a GO Term analysis of exclusive proteins at each sampling time was performed to compare accurately the natural behavior of the microbiota. A total of 259 (FW_EL: 124; FW_UL: 135) and 200 (B_EL: 158; B_UL: 42) exclusive proteins were identified and detailed in **Supplementary File 3**. As previously mentioned, this analysis is mostly focused on the major amount of the metaproteome.

At the end of the loading phase for the fine wine profile (FW_EL), the metabolism of amino acids, mostly aminoacyl-tRNA ligases, cell division, and, briefly, stress-related response (metabolism of glutathione and chaperones) was highlighted between most abundant species. At the end of the exhausting phase (FW_UL), the formation of peptide release factors, ribosomal subunits, and stress-related response (chaperones, redox activity, and synthesis of lipopolysaccharides) were some of the most reported GO Terms. The end of the loading phase for the beer profile (B_EL) showed the metabolism of amino acids, energy metabolism pathways, and redox activity as main functions; exclusively identified in *K. europaeus*, outer membrane proteins as ABC transporters, and porins. At the end of the exhausting phase (B_UL), the predominant species were involved in ATP-binding, redox processes, and cellular homeostasis while the minor fractions were in stress-response (chaperones). *K. europaeus* (73.5%) not only shared the main GO terms with other related and minor species but was involved in other exclusive ones. A quantitative proteomic description of this species could provide an accurate approach to the microbiota function under the comparison of two acetification profiles.

Comparative of Two Quantitative Proteomic Profiles in *Komagataeibacter europaeus*

After subjecting all the samples to an LC/MS^2 enrichment analysis, a total of 1,533 valid proteins were identified in *K. europaeus*. From them, 1,420 (B_EL: 1,264; B_UL: 1,245; FW_EL: 1,121; FW_UL: 1,152) were found in at least 50% of samples in one cycle time. The distribution of these proteins throughout the phases of two acetification profiles was summarized in an intersection plot shown in **Figure 2A**. Of the 1,420 proteins, 950 (66.9%) were common throughout both profiles, with 174 (12.3%) exclusive of the beer profile, and 90 (6.3%) of the fine wine profile. The amount of exclusive proteins at each sampling time was considerably minor: 39 out of 1,420 (2.7%) exclusive proteins at FW_UL were highlighted against 21 (1.5%) at B_EL, 6 (0.4%) at FW_EL, and 5 (0.4%) at B_UL. Then, 9 out of 1,420 (0.6%) proteins were found exclusively at the end of the loading phase (EL) and 6 (0.4%) before unloading (UL). The results evidenced that an important amount of the *K. europaeus* proteome was stable not affected by the change of phase or raw material.

Protein Clustering Analysis: Quantification Patterns and Interaction Networks

The proteome of *K. europaeus* was grouped according to the quantification pattern of each protein throughout each acetification profile. First, each protein quantification value in at least 50% of samples in all sampling times was normalized by z-score transformation and then clustered according to its pattern. **Figure 2B** shows a heatmap that summarized the

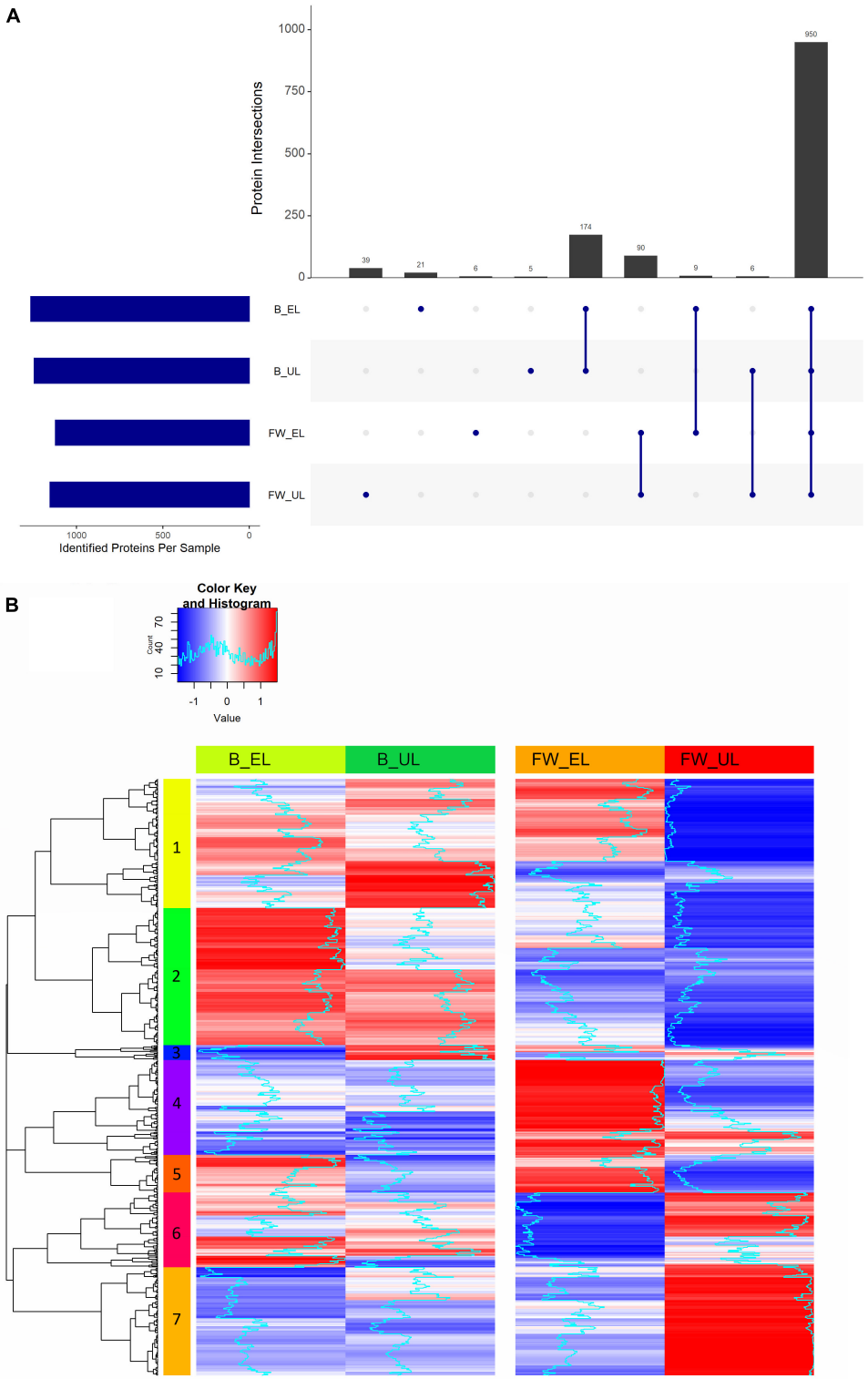

FIGURE 2 | (A) Intersection diagram of the proteins of *K. europaeus* identified in at least 50% of total samples in at least one phase. The number of proteins from each intersection group is represented on the bars. **(B)** Heat map showing the analysis of hierarchical clustering of the proteins identified in at least 50% of total samples in each phase. The number on each color represents each of the seven built clusters. Fine wine (FW), beer (B), end of loading (EL), just before unloading (UL).

hierarchical clustering carried out including a total of 832 proteins classified into seven clusters with different quantification patterns (more details can be found in **Supplementary Table 2**). Cluster 1 (n = 180) was characterized by a changing pattern throughout acetification of beer, but a marked decrease at the end of the exhausting phase in the fine wine vinegar (FW_UL). Cluster 2 (n = 191) was increased at the end of the loading phases, above all at B_EL, where quantification peaks were observed. Cluster 3 (n = 21), composed of a poor number of proteins, showed quantification peaks at B_UL. Clusters 4 (n = 132) and 5 (n = 53) were strongly upregulated at FW_EL, and also, Cluster 4 was decreased in the beer profile. Cluster 6 (n = 104) showed a changing pattern in the beer profile while in the fine wine profile, an increase just before unloading (FW_UL) was appreciated as in Cluster 7 (n = 151), where the quantification peaks were strongly observed (FW_UL).

Proteins from each cluster were then subjected to a protein-protein interaction analysis using the database STRING v11.0 to clarify the most relevant metabolic pathways related to each quantification pattern. **Figure 3** shows INM built from each cluster (six out of seven are represented), and those showed more interactions than expected (PPI enrichment p-value < 0.05):

- INM 1 (82 edges; PPI enrichment p-value, 1.71×10^{-13}) (**Figure 3A**) showed a high number of proteins related to the biosynthesis of amino acids (yellow nodes), mostly, L-glycine, L-serine, L-threonine, and L-lysine. A group of proteins at the top-left exhibited these proteins also involved in energy metabolism pathways [glycolysis (red nodes) and TCA cycle (purple nodes)]. Proteins related to the metabolism of purines (blue nodes) were found attached to it. Also, most of the alcohol dehydrogenase [ADH] subunits were classified in Cluster 1, even interaction groups that were not built (see **Supplementary Table 2**).
- INM 2 (123 edges; PPI enrichment p-value, 1.83×10^{-07}) (**Figure 3B**) exhibited a high number of proteins for the biosynthesis of aromatic amino acids (yellow nodes) (L-phenylalanine, L-tryptophane, and L-tyrosine), see middle-left group. Proteins related to the TCA cycle (red nodes) and the pyruvate metabolism (purple nodes) were also shown and connected to fatty acid biosynthesis proteins (light brown nodes), see middle and bottom-right groups. The metabolism of purines (blue nodes) and pyrimidines (light blue nodes) were observed evidencing a similarity to INM 1, although some particular groups were appreciated as biosynthesis of peptidoglycans (dark green nodes) and proteins (pink nodes), see bottom-left and top-right groups.
- INM 3 was not built because did not reach a PPI enrichment p-value < 0.05.
- INM 4 (86 edges; PPI enrichment p-value, 1.77×10^{-04}) (**Figure 3C**), similar to the previous INMs, showed groups relating processes like metabolism of amino acids (yellow nodes) to energy metabolism [TCA cycle (red nodes), pyruvate pathway (purple nodes)] and the purine (blue nodes) to pyrimidine metabolism (blue light nodes). Particularly, a group relating the biosynthesis of proteins (pink nodes) to aminoacyl-tRNA ligases (green nodes) was seen at the down-right.
- INM 5 (28 edges; PPI enrichment p-value, 2.95×10^{-4}) (**Figure 3D**) following the trend of previous INMs, predominated biosynthesis of amino acids (yellow nodes) (L-alanine, L-aspartate, and L-glutamate), proteins (pink nodes), metabolism of purines (blue nodes), and aminoacyl-tRNA ligases (green nodes).
- INM 6 (227 edges; PPI enrichment p-value, 1.00×10^{-16}) (**Figure 3E**) and INM 7 (353 edges; PPI enrichment p-value, 1.00×10^{-16}) (**Figure 3F**) presented both a highlighted central group composed mainly of ribosomal subunits, initiation, and elongation factors (pink nodes); ribosomal silencing and maturation factors were also found in INM 7. Around and/or attached to the central protein group, chaperones (dark green nodes), stress-response proteins (oxidoreductases, metabolism of glutathione, aldehyde dehydrogenase [ALDH] subunits, and outer membrane efflux pumps) (light brown nodes) were represented.

Differential Expression Analysis by Pairs: ANOVA and HSD Tukey's Test

A total of 141 proteins surpassed the statistical cut-off evidencing significant differences of quantification values in at least one pair comparison according to HSD Tukey's test corrected by multiple testing (q-value < 0.05) and $\log_2$ fold change in absolute value (FC) > 1: one protein for the pair B_UL/B_EL, 23 proteins for the pair FW_EL/B_EL, 76 for the pair FW_UL/B_UL, and 108 for FW_UL/FW_EL (see **Supplementary Table 3**). From these proteins, those that showed a strong significance (Tukey corrected by q-value < 0.01 and FC > 2) were represented in a radar chart and will be described below (see **Figure 4**). This Figure is an easy way to visualize the relationship between proteins and their abundance in each sample so that it can be quickly seen that the protein profile depends on both the sampling time (EL, UL) and the raw material (FW, B). Then, a detailed description of these protein groups can be carried out.

First, proteins that exhibited quantification peaks at B_EL (green) were mostly shown at the top-right of the radar chart. As observed in the corresponding clusters and INMs (1 and 2; see **Figures 2B**, **3A,B**) these proteins were involved in the metabolism of amino acids [glutathione reductase (gorAp), N-succinyl-transferase (dapDp)], of purines [adenine deaminase (adep)], aminoacyl-tRNA ligases [phenylalanine-tRNA ligase (pheSp)], and biosynthesis of proteins [riboflavin biosynthesis protein (ribFp), RNA polymerase sigma factor [RPOD] (rpoDp)]. Between them, rpoDp (cluster 1) was strongly down-regulated in the pairs FW_UL/B_UL and FW_UL/FW_EL (FC $\approx$ 3). One single protein presented the quantification peak at B_UL (yellow), DNA topoisomerase IV (parCp; cluster 1), essential in the segregation of chromosomes during DNA replication, especially down-regulated in the pair FW_UL/B_UL (FC $\approx$ 3).

Next, many proteins showed quantification peaks at FW_EL (blue), see clusters and INMs 4 and 5 (**Figures 2B**, **3C,D**): acetolactate synthase [large subunit] [ALS], involved in the

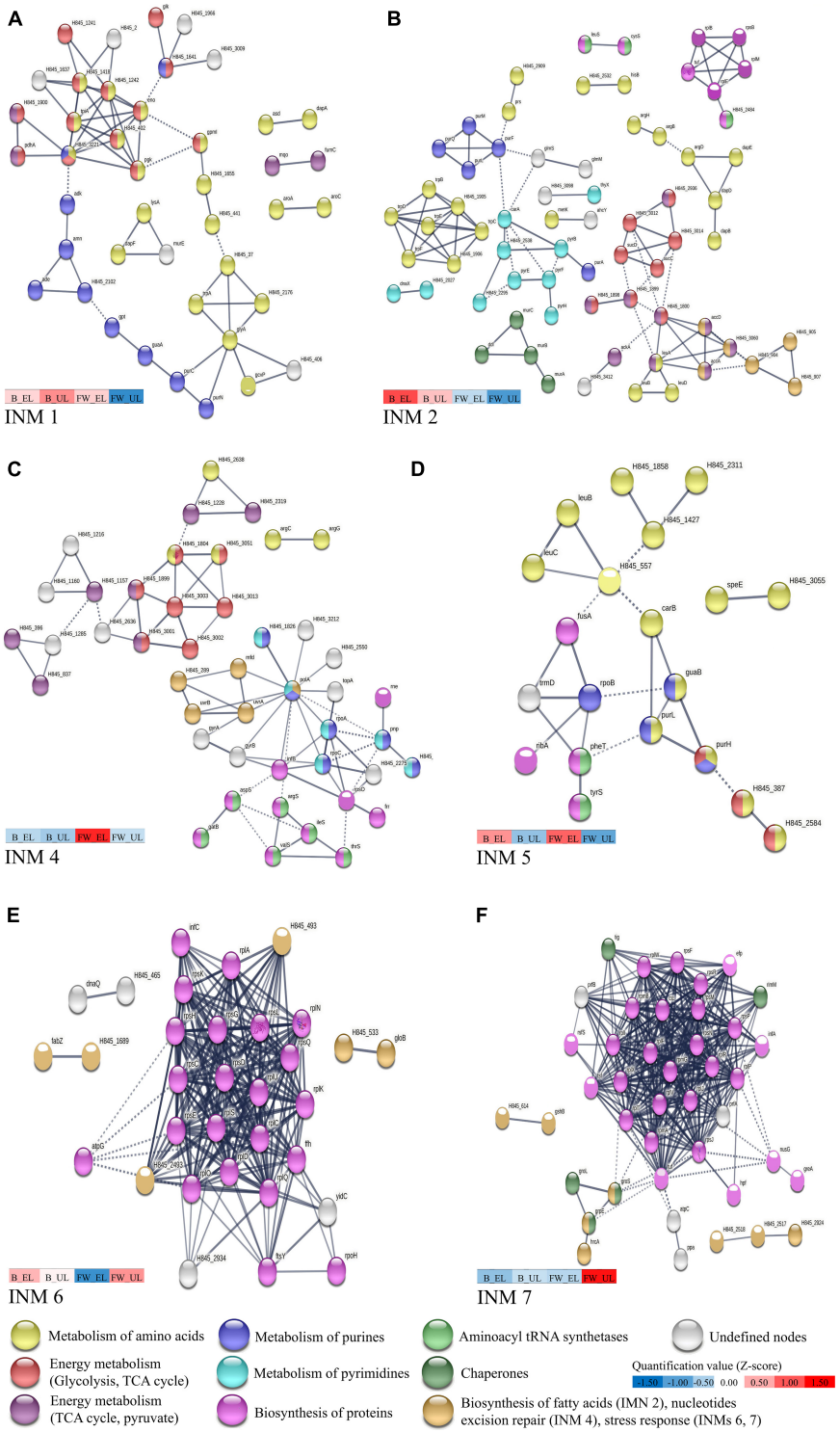

FIGURE 3 | High confidence protein-protein interaction network maps (INM) performed in *K. europaeus* of proteins from each cluster, shown in **Figure 2B**, with a PPI enrichment *p*-value < 0.05 using STRING v11.0. **(A)** INM 1 (Cluster 1), **(B)** INM 2 (Cluster 2), **(C)** INM 4 (Cluster 4), **(D)** INM 5 (Cluster 5), **(E)** INM 6 (Cluster 6), and **(F)** INM 7 (Cluster 7). Proteins are shown as nodes and interactions between them are represented by edges whose thickness indicates the strength of each interaction. Nodes with the same color represent a specific function based on protein annotations according to the databases Uniprot and KEGG. A color scale showing the mean quantification values (z-score) of clustered proteins (see **Figure 2B**) that compose each INM at each sampling time is represented. Undefined nodes (gray color) belong to proteins with a low prevalent or unknown function. *K. xylinus* E25 was used as a model organism due to the high homology (MUM index of 0.21 with the genome of *K. europaeus*; Ryngajłło et al., 2018). The list of total proteins subjected to STRING analysis and their respective annotations can be found in **Supplementary File 2**.

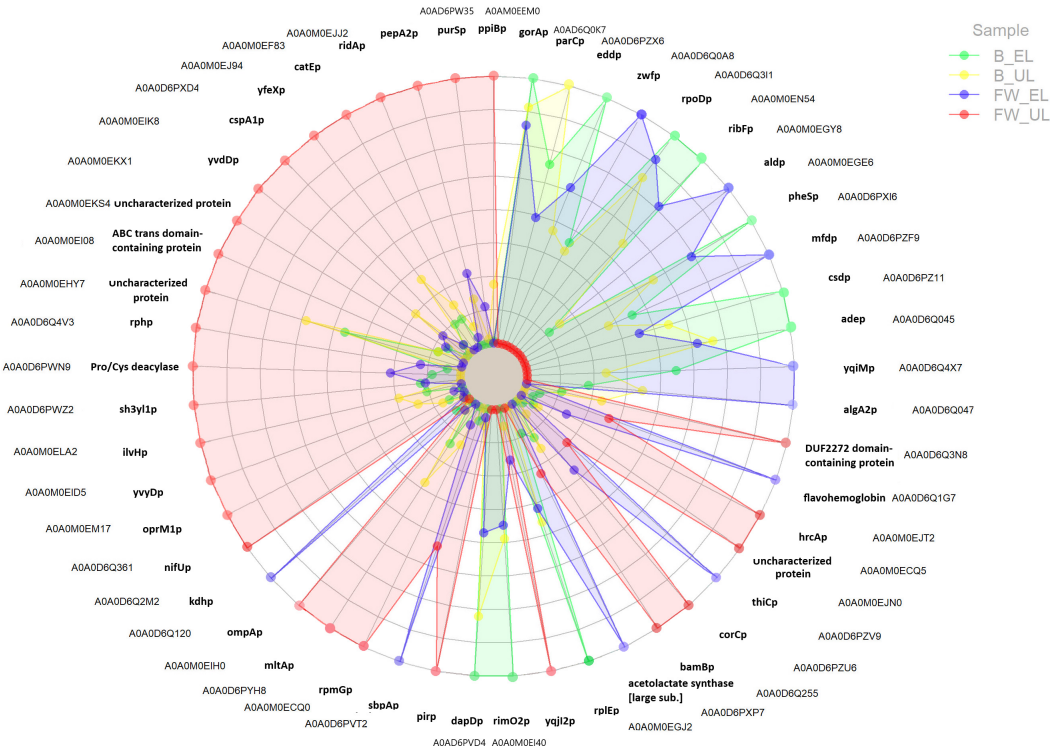

FIGURE 4 | Radar chart representing proteins showing the strongest significative differences according to HSD Tukey's test corrected by multiple testing (q-value < 0.01) and $\log_2$ fold change (in absolute value) > 2 (see **Supplementary Table 3**). The quantification area of each protein in each profile (FW, fine wine; B, beer) and sampling time (EL, end of loading; UL, just before unloading) is included.

biosynthesis of branched-chain amino acids (BCAA); other proteins related to energy metabolism activity like the pentose phosphate pathway (PPP) [glucose-6-phosphate dehydrogenase [GPDH] (zwfp)] and the TCA cycle [α-ketoglutarate dehydrogenase [αKDH] (kdhp), 3-succinoyl-semialdehyde dehydrogenase [SSADH] (aldp)]; flavohemoglobin and flavin oxidoreductase [NADH] (yqiMp), known to be involved in the biosynthesis of flavoproteins that catalyzes oxidoreduction processes while sulfate-binding protein (sbpAp) and phospho-methyl-pyrimidine synthase [THIC] (thiCp) may provide FeS clusters for the electron transport chain. Most of these proteins were upregulated in the pair FW_EL/B_EL and downregulated in FW_UL/FW_EL; for the first one, thiCp was remarked (FC = 4.68). Although the aforementioned proteins maintained acceptable levels of expression in the phases described (B_EL, B_UL, and FW_EL), all of them were characterized by a significant decrease at FW_UL (red).

A total of 28 proteins, mostly distributed at the half left of the radar chart (see **Figure 4**), were characterized by significant quantification peaks at FW_UL (red) against a marked decrease in the rest of the phases, see clusters and INMs 6 and 7 (**Figures 2B**, **3E,F**), and most of them were upregulated in FW_UL/B_UL and FW_UL/FW_EL pairs. Nitrogen-fixing thioredoxin (nifUp), iron-binding nuclear pirin (pirp), dehydrogenase PQQ (bamBp), and glyoxalase resistance protein (catEp) are related to oxidoreductase activity and maintain the redox balance. The outer membrane proteins ompAp and oprM1p, acting as porin of small solutes and efflux transport pump, respectively, and both playing a role in the outer membrane stability and resistance to environmental stress, were upregulated in the pair FW_UL/FW_EL (FC = 3.64 and 3.13 respectively). Other transmembrane proteins as ABC transporter (FC = 4.17) and murein hydrolase A [MLTA] (mltAp) (FC = 2.60) were then upregulated in the aforementioned pair while other proteins were related to the regulation of the translation: endoribonuclease [L-PSP] (ridAp), ribonuclease [RPH] (rphp), ribosome hibernation promoting factor [HPF] (yvyDp), and cold-shock protein [CSPA] (cspA1p). Acetolactate synthase [ILVH] (ilvHp) was shown, as occurred at FW_EL, while phospho-ribosylformylglycinamidine synthase [PURS] (purSp) catalyzed the first steps of the biosynthesis *de novo* of purines, and was one of the most upregulated proteins (FC = 5.58).

DISCUSSION

This study focused on the analysis of the acetification of two different substrates, aimed to delve into the behavior of the bacteria responsible for the process when the nutritional profile of the medium offers significant differences due to its

composition, especially, in regard to the availability of carbon sources additional to ethanol, namely carbohydrates. Indeed, if the microbiota responsible is able to adapt to the conditions of the environment by modifying its metabolism and taking advantage of the resources available in each case, it would be a proof of its great versatility and therefore, survive in different and particularly, aggressive environments. In previous studies by the authors, qualitative and quantitative proteomic analysis of one synthetic alcoholic medium acetification process were carried out. Now, a new study is being conducted for much more complex media (fine wine and a craft beer) from which the differences in proteomic profiles are being disclosed. Then, a novelty from this study is that even when using different raw materials, the microbiota composition is similar, but its metabolism, at a proteome level, is different. Next, a detailed discussion about these issues will be carried out while the main differences between both profiles and approached proposals about the metabolic differences are made.

As it is known in the vinegar industry, the total strength of the medium (ethanol plus acetic acid concentration), which remains constant throughout the cycle, can affect to the cell activity and concentration (García-García et al., 2007; Baena-Ruano et al., 2010). In the present study, a mild environment offering no special stressing conditions has been used to study some basic aspects of the complex microbiota of the process. Here, both media showed an initial ethanol concentration of around 10% (v/v), and the acetic acid level could be disregarded (see section "Raw Material"). The initial total strength [% (w/v) of acetic acid plus % (v/v) of ethanol] is 10 total degrees. Then, in each cycle, 3.5/4 L of medium containing 9.2 [7.9 $\pm$ 0.2% (w/v) plus 1.3 $\pm$ 0.3% (v/v)] and 8.0 [6.8 $\pm$ 0.7% (w/v) plus 1.2 $\pm$ 0.1% (v/v)] total degrees for fine wine and beer profiles, respectively, are unloaded. The differences between the initial total strength and unloaded product appear due to the volatile losses (around 8 and 20% in each medium, respectively). The foaming generated in the beer medium would favor the volatile losses (20%) and no special care was taken to avoid these losses since it would not affect the aim of the work. Regardless, 92 and 80% of the disappeared ethanol was used for acetic acid formation and the rest was stripped by air or transformed by bacteria for other uses (Jiménez-Hornero et al., 2020).

Regarding the protein composition of the microbiota, no relevant differences were appreciated between the sampling times of each acetification profile. This composition showed a strong similarity to results obtained in our previous work that characterized an alcohol vinegar profile (Román-Camacho et al., 2021). These results may be explained by the operating mode followed in this work in which the same starter culture, consisting of a mixed broth coming from the aforementioned alcohol medium acetification, concretely, from the end of the ethanol exhausting phase, was used for both acetification profiles. Under these working conditions, in which the fine wine and then, beer acetification were consecutively performed, the raw material change might not modify excessively the starter microbial composition despite the additional nutritional richness that these natural substrates might provide. Subsequently, the main functions of the exclusive proteins in each phase were detailed to compare the microbiota activity in each acetification profile. The predominant species of the microbiota exhibited a natural behavior according to other authors that worked using submerged biotransformation (Fernández-Pérez et al., 2010; Qi et al., 2014; Trček et al., 2016) while the minor species showed a high-stress response, probably trying to coexist along with the better-adapted ones. Even if the protein amount that provides each species affects its role in the metaproteome, all of them might participate in the whole function of the microbial community (Peng et al., 2021). *K. europaeus*, supplying a mean frequency of 73.5%, far above the rest, not only shared the main GO Terms with other species but was involved in other exclusive ones. It is worth noting that this species was also the most representative in our previous studies (Román-Camacho et al., 2020, 2021). Therefore, a quantitative proteomic description of *K. europaeus*, comparing two acetification profiles, might provide a prediction of the microbiota role and characterize the natural raw materials used.

K. europaeus is well-known as one of the main microorganisms responsible for industrial vinegar production. High ethanol-oxidizing ability, acetic acid requirement, and tolerance to high acidity levels [10–20% (w/v)] determine its suitability for this biotransformation (Trček et al., 2007; Yamada et al., 2012; Gullo et al., 2014). These capabilities allow it to perform an efficient incomplete oxidation reaction of the ethanol into acetic acid. This particular metabolic process consists of a two-step reaction (see **Figure 5**). First, alcohol dehydrogenase (ADH) binds to pyrroloquinoline quinone (PQQ) to oxidize the ethanol into acetaldehyde. Next, acetaldehyde is oxidized to acetic acid by aldehyde dehydrogenase (ALDH); both enzymes are located on the periplasmic side of the inner cell membrane (Adachi et al., 1980; Ameyama and Adachi, 1982). Further, NAD^+ and $NADP^+$ may be used as coenzymes by ADH-NAD and ALDH-NADP, located in the cytoplasm (Qin et al., 2021; Sriherfyna et al., 2021). The acetic acid produced at the periplasm is released into the medium increasing its external concentration which, in turn, triggers its diffusion and accumulation in the cytoplasm (Gullo et al., 2014; Qiu et al., 2021). The TCA cycle may assimilate the inner acetic acid through the input of acetyl-CoA providing biosynthetic precursors of amino acids and nucleic acids thus replenishing cell material throughout the loading phase and early stages of the ethanol depletion phase. The use of raw material with sugar content, as is the case of our craft beer, can lead to assimilating firstly, the available glucose and draining biosynthetic precursors directly from energy metabolic pathways as the PPP and the glycolysis. At the final moments of acetification, cells would trigger different membrane mechanisms dependent on proton motive force for the acetic acid release and detoxification. This molecular strategy, proposed in the present work, would allow *K. europaeus* to prevail over other species during the acetification process. These findings will be exhaustively detailed in the rest of the discussion based on hierarchical clustering, protein-protein interactions, and statistical analysis. Furthermore, it has been sectioned to facilitate the understanding of these microbial behavioral aspects at a quantitative level. In short, the discussion has been organized by analyzing the results obtained for the

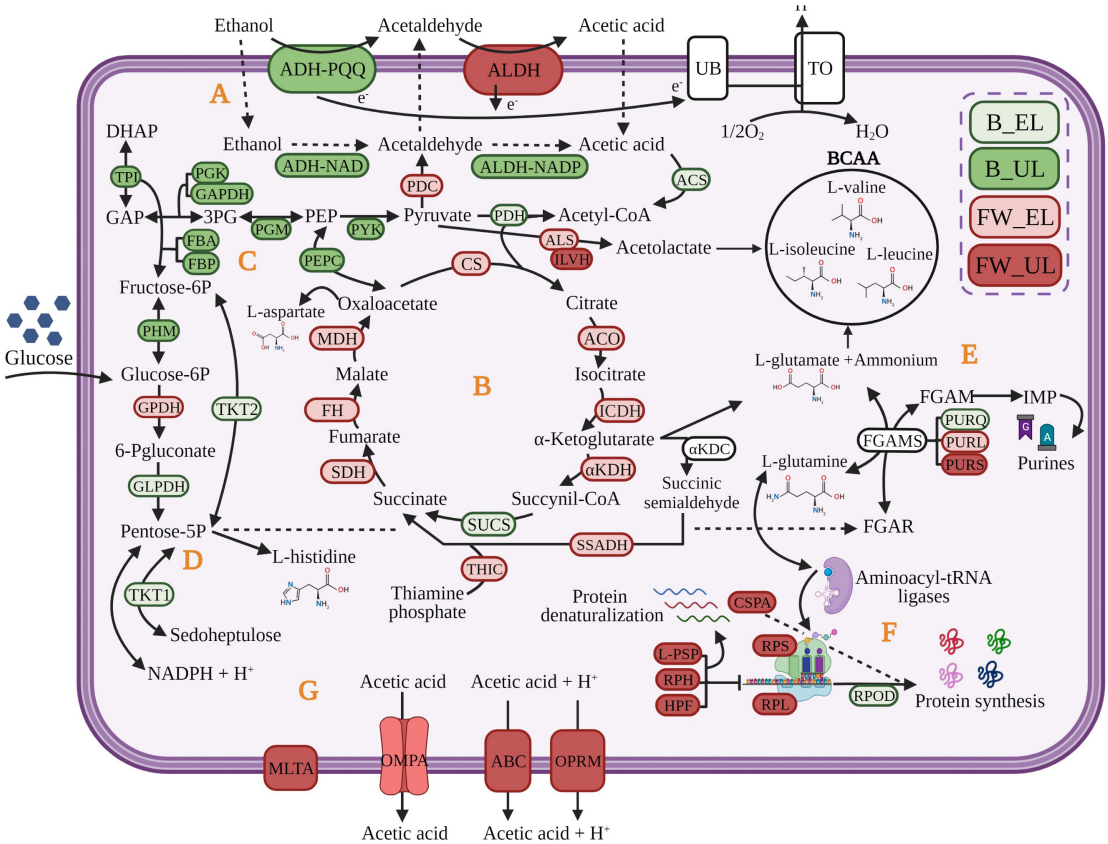

FIGURE 5 | Molecular strategy of *K. europaeus* proposed to prevail throughout acetification process. The colors of the proteins represent the phase in which the protein had the highest quantification value. Fine wine (FW), beer (B), end of loading (EL), just before unloading (UL). **(A)** oxidation of ethanol into acetic acid; **(B)** TCA cycle; **(C)** glycolysis; **(D)** pentose phosphate pathway; **(E)**, amino acids and purines formation from biosynthetic precursors; **(F)** regulation of protein synthesis; **(G)** membrane mechanisms for acetic acid release. GAP, glyceraldehyde 3-phosphate; DHAP, dihydroxyacetone phosphate; 3PG, 3-phosphoglycerate; PEP, phosphoenolpyruvate; FGAR, formylglycinamide ribonucleotide; FGAM, formylglycinamidine ribonucleotide; IMP, inosine monophosphate. The definitions of the protein abbreviations and their respective highest quantification values are given (see **Supplementary Table 4**). Created with BioRender.com (with publication license).

two raw materials as a whole according to the most relevant metabolic processes; and in this way, the differences existing in both acetification profiles can be better appreciated.

The Essential Role of the Biosynthesis of Amino Acids and Nucleic Acids From Metabolic Precursors Replenishing Cellular Material Losses

The metabolism of amino acids seems to be one of the most representative metabolic pathways of *K. europaeus*, as can be appreciated in most protein clusters, above all, in those showing quantification peaks at the end of the loading phase (Clusters 1, 2, 4, and 5). The amino acids are synthesized from intermediaries of the TCA cycle, the glycolysis, and the PPP through L-glutamate and L-glutamine, both acting as nitrogen sources that are self-regulated according to the cell requirements (Yin et al., 2017; Sankuan et al., 2020). These results suggest that AAB might use their high nitrogen recovery capability to convert continuously nitrogen sources like proteins, nucleic acids derived from raw materials, and apoptotic cells into amino acids and ammonium thus replacing their consumption and cell material losses during the loading phase (Álvarez-Cáliz et al., 2012; Kuypers et al., 2018). In this sense, ALS is related to the synthesis of BCAA and L-valine, L-leucine, and L-isoleucine, from pyruvate, are strongly upregulated at FW_EL. It is also interesting to note that ILVH was highly upregulated at FW_UL. BCAA may provide NH_3 and energy to neutralize the increase of acid final products during the exhausting phase and support intracellular pH balance through deamination, as proposed by other authors who reported different isoforms of acetolactate synthase in diverse acidophilic organisms (Santiago et al., 2012; Andrés-Barrao et al., 2016; Yin et al., 2017). Our findings supported the essential role of the metabolism of amino acids throughout acetification and especially, suggest the addition of BCAA to the fermentation culture as a possible system to protect the cellular integrity and increase productivity.

Conversely, the biosynthesis *de novo* of purines and pyrimidines requires the addition of amino acids to the pentose-5-phosphate, coming from the PPP, and metabolic

energy (ATP) as can be observed in INMs belonging to clusters showing quantification peaks at EL (1, 2, 4, and 5). To our knowledge, this pathway has been barely researched in AAB, but here, phosphoribosyl-formyl-glycinamidine synthase complex (FGAMS) has been found (see **Supplementary Table 2**). FGAMS, composed of three subunits (PURQ, PURL, and PURS), carries out the ATP-dependent formation of formyl-glycinamidine ribonucleotide [FGAM] converting L-glutamine to L-glutamate (Tanwar et al., 2012). In previous works characterizing an alcohol vinegar microbiota (Román-Camacho et al., 2020, 2021), some GO Terms related to the synthesis of organic heterocyclic aromatic compounds, concretely, nucleic acids were identified. In the present work, the main subunits showed quantification peaks at different phases (PURQ, B_EL; PURL, FW_EL; PURS, FW_UL) although along with others, most of them were highlighted at the end of the loading phase. This fact could indicate that the synthesis of nucleic acids is integrated with other metabolic pathways, being part of a biological system that aims to replenish cellular material losses caused after unloading, improving adaptability, and ensuring the survival of the microbiota, above all, *K. europaeus*.

The TCA Cycle as a Key Pathway in the Cytoplasmatic Acetic Acid Assimilation and Biosynthetic Precursors Source

The TCA cycle has been studied exhibiting an important function in the metabolism of AAB (Nakano and Fukaya, 2008; Kwong et al., 2017). The protein groups involved in this pathway were predominant in clusters whose quantification patterns were higher at EL phases (Clusters 1, 2, and 4). All the TCA cycle enzymes were found in the proteome of *K. europaeus* (see **Supplementary Table 2**) and all of them were downregulated, mainly in the fine wine profile (see **Figure 5**). In this work, αKDH, SSADH, and THIC were highlighted so they might play a critical role. Zhang and Bryant (2011) and Lei et al. (2018) investigated that αKDC and SSADH might form succinic acid via succinic semialdehyde by using cofactors of thiamine phosphate in *Synechococcus* sp. PCC7002. The TCA cycle can supply α-ketoglutarate to the synthesis of L-glutamate that provides amino groups in biosynthetic reactions, besides oxalacetate. For this purpose, the acetyl-CoA is provided to the TCA cycle by the conversion of pyruvate obtained in the glycolysis and of acetic acid derived from the ethanol oxidation (Mamlouk and Gullo, 2013; Qin et al., 2021). Because of the direct drain of intermediates from the TCA cycle to biomass, amino acids are partly derived from ethanol (Adler et al., 2014). In this sense, considering that AAB must cope with constant changes in ethanol, acetic acid, and cellular concentration because of the semi-continuous state of the cycles in our experiment, we suggest that the TCA cycle might be used for assimilating cytoplasmatic acetic acid, coming from ethanol, supplying energy, and biosynthetic precursors according to other authors (Ramírez-Bahena et al., 2013; Adler et al., 2014; Andrés-Barrao et al., 2016; Zheng et al., 2017).

The Pentose Phosphate Pathway and Glycolysis Are Used to Assimilate the Available Glucose Obtaining Rapidly Biomass and Energy for the Synthesis of Precursors

The pentose phosphate pathway (PPP) is the main metabolic route of AAB to incompletely oxidize the glucose of the medium providing several precursor metabolites, mainly pentose-5-phosphate, necessary for the biosynthesis of amino acids (L-histidine) and nucleic acids (Adler et al., 2014; García-García et al., 2017). Several authors have related the enhance of PPP to the generation of NADPH + H^+, also involved in biosynthetic processes even in reducing oxidative stress (Yin et al., 2017; Christodoulou et al., 2018; Sriherfyna et al., 2021). Although many of the species of *Acetobacter* and *Komagataeibacter* have demonstrated a higher preference for ethanol as a carbon source, in this analysis, most PPP enzymes were expressed when the ethanol concentration was higher (EL phases) (see **Supplementary Table 2**). Except for GPDH, which was strongly expressed in the fine wine profile (FW_EL), the rest of the PPP enzymes were higher quantified in the beer profile (B_EL) (see **Figure 5**). The remaining sugar content of the beer (7% before dilution) may provide glucose as a carbon source allowing AAB, mainly *K. europaeus*, to rapidly obtain biomass and energy for the synthesis of precursors (García-García et al., 2017; Qin et al., 2021). Indeed, the glycolysis enzymes were also higher expressed in the beer profile, in this case, and mostly upregulated (B_UL). Zheng et al. (2017) study showed that growing *A. pasteurianus* in a medium containing 1% initial acetic acid, PPP was decreased, and energy metabolism was enhanced by the production of pyruvate. Despite its ethanol preference, *K. europaeus* might assimilate, firstly, the glucose in the beer medium for rapid biosynthesis of precursors, which are not possible to obtain by other pathways, obtaining energy, and thus, prevail over other species that exhibit high glucose preference. This fact would explain the presence in our results of numerous protein groups involved in these pathways in clusters whose patterns showed quantification peaks during beer acetification (Clusters 1, 2, 4, and 5).

The Biosynthesis of Ribosomes and Proteins Is Regulated With the Increase of Acetic Acid Concentration

The biosynthesis of proteins has been reported by different authors as one of the most highlighted metabolic pathways in AAB throughout the acetification process (Andrés-Barrao et al., 2012; Xia et al., 2016; Román-Camacho et al., 2021). Here, the most of functional groups involved in this process, mainly composed of ribosomal subunits, showed quantification peaks at FW_UL (Clusters 6 and 7). However, few of them were significantly upregulated since proteins that surpassed the statistical cut-off were related to the regulation of the translation. Among them, L-PSP inhibits the synthesis of proteins by the degradation of mRNAs, HPF dimerizes the bacterial functional ribosomes into inactive 100S ribosomes (Matzov et al., 2019),

and RPH assists the maturation of tRNAs and the degradation of structured RNAs mainly in *E. coli* (Jain, 2012). Some authors have reported a decrease in the biosynthesis of proteins when the acidity levels increase in *A. pasteurianus* through functions as the recycling of ribosomes (Andrés-Barrao et al., 2012; Xia et al., 2016). Then, CSPA (see **Figure 5**), was strongly upregulated and its function has been discussed in *E. coli* as an RNA chaperone that prevents the protein refolding by ribonucleases (Rennella et al., 2017). When the acetic acid concentration is diluted during the loading phase the process of protein synthesis seems to occur efficiently through the presence of aminoacyl-tRNA ligases in Clusters 2, 4, and 5, binding tRNAs to specific amino acids and ensuring an accurate translation process (Román-Camacho et al., 2021). It is worth noting the drastic decrease of expression of initiation factors as RPOD with the increase of acetic acid level (FW_UL). In summary, these findings suggest that acetic acid accumulation generates a stress response thus regulating the formation of ribosomes and proteins.

Membrane Mechanisms of Response to Acetic Acid Stress Derived From the Incomplete Oxidation of Ethanol

The incomplete oxidation of the ethanol of the medium is carried out by membrane-bound systems directly coupled to respiratory chains and allowing that oxidation reaction to take place in the periplasm without a requirement of transport across the membrane (Qin et al., 2021; Qiu et al., 2021). In this work, numerous subunits of PQQ-ADH and ALDH were identified and mainly expressed at UL phases (see **Supplementary Table 2**), but in general, these enzymes were stable indicating that the oxidation of ethanol could be constantly active throughout acetification. The acetic acid produced in the periplasm is released into the medium, thus increasing its external concentration. However, when it occurs, this compound can diffuse and accumulate into the cytoplasm along with that generated inside by the activity of ADH-NAD and ALDH-NADP (Adler et al., 2014; Gullo et al., 2014). In this sense, some systems acting to detoxify the cell might be participating through the upregulated proteins at FW_UL. First, those related to redox homeostasis maintenance, particularly, implicated the maturation of iron-sulfur (FeS) clusters acting as cofactors in the electron transfer (nifUp), cell apoptosis (pirp), and detoxification (catEp) (Benoit et al., 2018). To our knowledge, this set of proteins had never been reported in AAB. Secondly, outer membrane proteins, as permeable porins of small solutes (OMPA) and efflux pumps (OPRM, putative ABC-transporter) (see **Figure 5**), might control the cellular output of acetic acid, whose concentration increases during the fermentation phase (Nakano and Fukaya, 2008; Confer and Ayalew, 2013). MLTA, participating in the maintenance of the peptidoglycan layer under these conditions, show even more evidence of the importance of the cell surface as an efficient mechanism against the acetic acid stress used by the species of *Komagataeibacter*, as other authors have well-studied (Wang et al., 2015; Andrés-Barrao et al., 2016).

CONCLUSION

A comparison of two acetification profiles using different raw materials was established to study the natural behavior of the involved microbiota through the metaproteome and, exhaustively, since it is the prevalent species, the quantitative proteomic profile of *K. europaeus*. Although the use of different raw materials seems not to affect the microbial composition, the microbiota behaved differently by significant changes in the expression of the proteome. In this work, it has been suggested that the inner acetic acid coming from the oxidation of ethanol might be assimilated in the TCA cycle providing biosynthetic precursors along with other metabolic pathways (PPP and glycolysis) if glucose is available, as is the case of one of the fermentation media studied (craft beer). These processes replenish the cell material losses (amino acids and nucleic acids) after unloading, throughout the loading phase. The excess of acetic acid in the cytoplasm would also be released to the medium by some cell membrane mechanisms proton motive-force dependent at the final stages of the acetification. This complete strategy has been reported in **Figure 5**, highlighting the phase at which each protein had a higher quantification value. The differences in the metabolic behavior throughout each acetification profile were more accentuated in the fine wine vinegar than in the craft beer vinegar. In this profile, FW_UL was a period significantly differentially based on statistical analysis. Metabolomic assays that would allow clarifying the differences between the associated metabolites to these raw materials, in more detail, are underway. These findings may lay the groundwork of a vinegar microbiota profile, at a protein level, under smooth operating conditions. Future studies might be undertaken to evaluate the effect on the microbiota of media with higher levels of ethanol and acetic acid and even comparatives studies to achieve a multi-omics integrative profile. This work might increase the knowledge of the use of diverse raw materials and optimize the operating conditions.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI—PXD031147.

AUTHOR CONTRIBUTIONS

JR-C: methodology, validation, formal analysis, data curation, writing—original draft preparation, and visualization. JM and IG-G: conceptualization, investigation, resources, writing—review and editing, supervision, project administration, and funding acquisition. IS-D: methodology, validation, formal analysis, conceptualization, and visualization. TG-M: conceptualization, data curation, validation, and supervision. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.840119/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

3.2. Chapter IV. Metagenomics and protein fingerprinting for the characterization of the microbiota of diverse vinegars obtained by submerged culture: the use of the "omics" tools 16S rRNA Amplicon Sequencing and MALDI-TOF MS

Once the microbiota present throughout the acetification of the three working media was described by metaproteomics (see section 3.1.), the confirmation of the previous results using other omics tools was considered necessary. In the present work, the composition of a starter microbiota propagated on and subsequently developing the three acetification profiles (synthetic alcohol-based medium, craft beer, and fine wine), was characterized and compared. For this purpose, a metagenomic tool, such as the 16S gene rRNA amplicon sequencing, and another proteomic tool based on the protein fingerprinting, such as matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS), were used. The combination of a culture-independent technique with a culture-dependent method was implemented for the first time to identify the microbiota members inhabiting the submerged vinegar production.

A total of 12,443 unique amplicon sequence variants (ASVs) were obtained of which, after clustering and chimera filtering, remained 6,187 as unique operational taxonomic units (OTUs) at 97% identity in, at least, one out of a total of 26 samples. A metagenomic qualitative analysis provided the number and distribution of unique OTUs within samples for each acetification process. The high number of OTUs of the starting inoculum samples was highlighted along with variations among the sampling times in the three acetification profiles. Higher biodiversity of OTUs was also appreciated in the inoculum samples. These findings evidenced that the nutritional composition of each raw material may influence the number and distribution of OTUs in the vinegar metagenome and, particularly, in the original inoculum before undergoing diverse environmental changes because of the switch to the different media. To perform a taxonomic study to facilitate an accurate identification of the microbiota, 16S rRNA amplicon sequencing and MALDI-TOF MS were applied. 16S rRNA amplicon sequencing revealed that throughout the time courses of the three acetifications, numerous taxa from 30 different phyla were found highlighting Proteobacteria and Fusobacteria, among others. Of them, the AAB genus *Komagataeibacter*, which dramatically outnumbered the rest of the taxa, and a minor fraction of microorganisms including the AAB genus *Acetobacter*, other bacteria groups (such as *Cetobacterium*, *Rhodobacter*, *Bacillus*, and *Sphingomonas*) and others belonging to archaea, mainly

Nitrososphaeraceae, were the main representatives. MALDI-TOF MS analysis allowed confirming the presence of *Komagataeibacter* in vinegar by the identification of the species *K. intermedius* in addition to reporting another bacteria species, *Lysinibacillus fusiformis*.

The use of the "omics" tools implemented in this work has allowed: first, to confirm the composition of the predominant microbiota obtained in our previous metaproteomic approaches (section 3.1.), mainly the AAB groups *Komagataeibacter* and *Acetobacter*; second, to identify some taxonomic groups never to date found in vinegar produced by the submerged culture method. However, further assays will be necessary to clarify the role of these microorganisms in the microbial community present during acetification; third, new insights on the characterization of the raw materials used have been obtained. This point again highlights the need for metabolomic studies to describe in more detail the differences in the composition of both the raw materials and vinegars during their submerged production (section 3.3.). The results obtained from this study may contribute to improving the understanding of the behavior of the vinegar microbiota and may have biotechnological interest in the vinegar-making industry.

This work has been accepted in the journal *Frontiers in Microbiology* with the title "*Combining omics tools for the characterization of the microbiota of diverse vinegars obtained by submerged culture: 16S rRNA Amplicon Sequencing and MALDI-TOF MS*", see section 6.1.1., appendix. It is currently in the production phase for online publication.

3.3. Chapter V. Study of the influence of the acetification process of synthetic (alcohol-based) and natural media (fine wine and craft beer) on the characterization of the vinegars "volatilome"

3.3.1. Introduction

The organoleptic properties of vinegar are influenced by acetic acid although many other metabolites are released into the medium because of the metabolic reactions taking place throughout the acetification process. Among them, the volatile compounds are essential precursors for the aroma that may be transferred from the raw material to the final product. In this work, the aim was to study the effect of the acetification of synthetic (alcohol-based) and natural (fine wine and craft beer) media on the whole of the minor volatile compounds or "volatilome" throughout the course of vinegar-making. For the characterization of the volatilome, minor volatile compounds were differentiated by gas chromatography coupled to mass spectrometry (GC-MS) both in the raw materials and phases of the elaboration of vinegar by submerged culture and working in a semi-continuous mode.

3.3.2. Materials and methods

Acetifications were performed from three different alcoholic substrates: first, a synthetic alcoholic-based medium (AW) and then, two natural raw materials, a dry fine wine (FW) from the Montilla-Moriles region (Bodegas Alvear S.A., Montilla, Córdoba, Spain) and a high-sugar craft beer (B) (Mahou-San Miguel, Córdoba, Spain). The first acetification (AW) was started using an inoculum consisting of a mixed culture coming from a fully active operating industrial tank (UNICO Vinagres y Salsas, S.L.L., Córdoba, Spain) making wine vinegar. A sample harvested at the final moments of this process was used as a starter culture for the subsequent acetifications (FW, B). The operating conditions were established according to the methodology developed by our group as can be found in our published works (see previous chapters of this report). Sampling was carried out at three different moments; first, directly from the raw materials without the inoculum, before starting the acetification (AW.S, FW.S, B.S); second, when the loading phase was finished, reaching the bioreactor working volume (AW.EL, FW.EL, B.EL); third, just before the unloading phase, when the acetic acid concentration was the highest throughout the cycle (AW.UL, FW.UL, B.UL).

The minor volatile compounds (< 10 mg/L) were identified and quantified in a two-step process following the methodology described by Dumitriu et al. (2020). The first step involved an extraction procedure by using a twister (0.5 mm film thickness and 10 mm length, Gerstel GmbH, Mülheim an der Ruhr, Germany) which was placed in a vial containing 10 mL of each 1:10 diluted sample and 0.1 mL of ethyl nonanoate (0.45 mg/L) as internal standard. After 100 min of stirring at 1500 rpm, the twister was removed and transferred into a desorption tube for chromatographic analysis. The second step involved the determination of the volatile compounds in a GC-MS equipped with a Gerstel TDS 2 thermodesorption system. Desorption tubes were heated at 280 ºC for 10 min to release the minor volatile compounds attached to the twister and retained in a TENAX adsorption tube. The adsorption tube was maintained at 25 ºC throughout the desorption and then, was subjected to a temperature increase until 280 ºC. GC-MS, equipped with an Agilent-19091S capillary column (30 m × 0.25 mm i.d. and 0.25 μm film thickness), was operated at 50 ºC for 2 min and then, until 190 ºC for 10 min. Helium was used as carrier gas at a flow rate of 1 mL/min. The mass detector worked in scan mode at 1850 V and checked the mass from 39 to 300 m/z.

The identification of the volatile compounds was carried out by using retention times of standards injected under the same chromatographic conditions, as the samples as well as the Wiley N7 spectral library. Quantification was performed using calibration curves of the standard.

STATGRAPHICS Centurion XVI.I software was applied to perform statistical analyses including the detection of significant differences by analysis of variance both univariate (ANOVA) and multivariate (MANOVA), hierarchical clustering (by Ward's method), and principal component analysis (PCA).

3.3.3. Results and discussion

3.3.3.1. Determination of minor volatile compounds

Minor volatile compounds of diverse vinegars were characterized and differentiated by performing qualitative and quantitative analyses. A total of 50 different minor volatile compounds were found: for the acetification samples of the synthetic alcohol-based medium (AW) were 24 (AW.S, 11; AW.EL, 21; AW.UL, 22; see Table 4) while for the

acetification samples of fine wine (FW) and craft beer (B), were 45 (FW.S, 39; FW.EL, 40; FW.UL, 39; see Table 5) and 47 (B.S, 46; B.EL, 39; B.UL, 39; see Table 6), respectively. Of the total, 9 volatile compounds were present in all samples throughout the three acetification processes and 22 in those of the final products (AW.UL, FW.UL, B.UL). Vinegar volatile compounds were distributed in 8 different chemical groups: acids, alcohols, aldehydes, ketones, esters, lactones, terpenes, and phenols.

Table 4. Minor volatile compounds identified in the samples of the acetification of the synthetic alcohol-based medium (AW). The letter shift throughout the process indicates significant differences at 95% confidence according to the analysis of variance (ANOVA) performed. AW.S, substrate; EL, at the end of the loading phase; UL, just before the unloading phase. **Source**: own work.

VOLATILE COMPOUNDS (µg/L)	AW.S			AW.EL			AW.UL		
	$\bar{x}$	σ		$\bar{x}$	σ		$\bar{x}$	σ	
ACIDS									
Pentanoic acid	0.00	0.00	C	547.60	51.51	A	428.66	23.30	B
Hexanoic acid	0.00	0.00	B	215.29	10.68	A	0.00	0.00	B
Octanoic acid	0.00	0.00	B	490.17	9.23	A	495.51	35.94	A
Decanoic acid	0.00	0.00	B	181.24	12.06	A	159.61	15.58	A
Dodecanoic acid	30.90	2.12	C	73.92	5.34	A	50.98	2.67	B
Tetradecanoic acid	176.50	10.61	A	143.53	10.19	B	109.26	8.50	C
Hexadecanoic acid	608.00	33.94	A	302.22	22.05	B	298.03	13.62	B
Octadecanoic acid	0.00	0.00	B	0.00	0.00	B	6.74	0.53	A
ALCOHOLS									
4-Vinylphenol	0.00	0.00	C	87.57	7.60	A	69.93	3.30	B
ALDEHYDES									
Benzaldehyde	0.00	0.00	C	310.45	24.19	B	911.58	91.09	A
Decanal	0.00	0.00	C	2.54	0.21	A	1.71	0.16	B
3,5-Dimethylbenzaldehyde	145.98	3.22	C	404.53	23.77	B	594.09	40.82	A
ESTERS									
Ethyl acetate	0.00	0.00	B	0.00	0.00	B	156.44	9.68	A
Isobutyl acetate	0.00	0.00	C	2.01	0.14	B	5.32	0.40	A
2-phenylethanol acetate	0.00	0.00	C	3.08	0.11	B	16.02	1.14	A
Ethyl butanoate	238.53	0.83	A	37.22	2.77	B	33.98	2.64	B
Ethyl pentanoate	12.02	0.33	A	0.00	0.00	B	0.00	0.00	B
Ethyl benzoate	18.73	0.23	B	18.20	1.26	B	26.33	0.53	A
Ethyl octanoate	1.73	0.13	A	1.72	0.11	A	1.70	0.14	A
Ethyl phenylpropanoate	9.97	0.86	C	114.35	10.28	B	161.73	14.85	A
Methyl trans-dihydrojasmonate	0.00	0.00	C	5.40	0.15	B	8.44	0.77	A
PHENOLS									
Guaiacol	0.00	0.00	B	37.99	2.36	A	42.85	3.59	A
LACTONES									
g-Nonalactone	5.65	0.51	A	6.02	0.21	A	6.15	0.30	A
TERPENES									
Limonene	22.14	1.09	B	25.59	1.59	A	23.97	0.51	AB

Table 5. Minor volatile compounds found in the samples of the acetification of fine wine (FW). The letter shift throughout the process indicates significant differences at 95% confidence based on the analysis of variance (ANOVA) performed. FW.S, substrate; FW.EL, at the end of the loading phase; FW.UL, just before the unloading phase. **Source**: own work.

VOLATILE COMPOUNDS (µg/L)	FW.S			FW.EL			FW.UL		
	$\bar{x}$	σ		$\bar{x}$	σ		$\bar{x}$	σ	
ACIDS									
Pentanoic acid	0.00	0.00	C	15181.92	710.40	B	34464.07	2557.85	A
Hexanoic acid	1116.88	101.82	C	1412.37	69.22	B	2121.92	129.36	A
Octanoic acid	10082.56	577.48	A	6428.55	303.00	C	7577.37	420.40	B
Decanoic acid	164.87	11.17	B	172.92	11.97	B	203.10	8.90	A
Dodecanoic acid	36.57	2.97	C	53.88	4.88	B	75.67	6.78	A
Tetradecanoic acid	79.43	3.42	C	121.18	9.55	B	182.47	4.65	A
Hexadecanoic acid	324.22	11.27	B	311.81	27.77	B	597.95	43.99	A
Octadecanoic acid	0.00	0.00	B	0.00	0.00	B	28.02	0.87	A
ALCOHOLS									
Isoamyl alcohols	407.85	29.76	A	160.22	13.39	B	83.77	6.83	C
Furfuryl alcohol	755.65	61.58	B	797.26	42.62	B	1560.71	115.92	A
Hexanol	1163.40	75.72	A	365.25	1.95	B	0.00	0.00	C
2-Phenylethanol	75.29	3.80	A	47.07	1.43	B	50.30	3.15	B
4-Vinylphenol	149.64	49.05	A	140.27	13.90	A	199.34	174.05	A
2-Methoxy-4-vinylphenol	81.31	52.93	A	45.22	2.25	A	51.89	3.00	A
ALDEHYDES									
Benzaldehyde	39.10	7.84	B	37.00	2.89	B	82.84	2.12	A
Phenylacetaldehyde	14.04	2.33	A	7.09	0.55	C	10.60	0.73	B
Octanal	0.77	0.09	A	0.00	0.00	B	0.00	0.00	B
Decanal	1.33	0.25	B	0.61	0.05	C	1.76	0.13	A
3,5-Dimethylbenzaldehyde	444.16	1.08	B	677.09	47.96	A	743.59	60.24	A
KETONES									
6-Methyl-5-hepten-2-one	0.00	0.00	A	9.62	0.20	B	15.90	0.67	C
ESTERS									
Ethyl acetate	64.74	4.75	A	43.83	2.78	B	50.31	3.84	B
Isobutyl acetate	5.20	0.52	C	145.40	12.33	B	269.93	21.56	A
Hexyl acetate	0.00	0.00	B	15.62	1.10	A	0.00	0.00	B
2-phenylethanol acetate	171.65	2.45	C	1652.71	164.05	B	2706.41	255.03	A
Ethyl propanoate	228.84	15.37	A	175.30	10.54	B	140.73	8.12	C
Ethyl isobutyrate	15.27	0.66	B	21.12	0.21	A	19.48	1.25	A
Ethyl butanoate	348.46	30.84	A	93.26	1.94	B	55.88	4.96	B
Ethyl hexanoate	363.58	34.81	A	0.00	0.00	B	0.00	0.00	B
4-OH-ethyl butanoate	7.94	1.36	A	3.10	0.30	B	2.06	0.13	B
4-OH-ethyl hexanoate	19.04	0.71	A	13.18	0.89	B	14.38	1.31	B
Ethyl benzoate	28.88	5.12	A	19.75	1.10	B	19.11	1.59	B
Diethyl succinate	51843.90	4898.5	A	28015.37	1315.05	B	28096.73	2294.46	B
Ethyl octanoate	56.60	0.57	A	4.64	0.17	B	3.96	0.31	B
Ethyl phenyl acetate	210.94	8.73	B	848.53	38.15	A	946.79	92.26	A
Ethyl phenylpropanoate	0.00	0.00	B	0.00	0.00	B	6.68	2.20	A
Ethyl isopentenyl succinate	3476.39	259.96	A	835.64	10.06	B	330.74	8.72	C
2-OH-3-ethyl phenylpropanoate	155.10	2.14	A	96.73	9.22	B	105.25	4.94	B
Methyl trans-dihydrojasmonate	6.09	0.21	A	5.66	0.54	A	6.52	0.40	A

VOLATILE COMPOUNDS (µg/L)	FW.S			FW.EL			FW.UL		
	$\bar{x}$	σ		$\bar{x}$	σ		$\bar{x}$	σ	
PHENOLS									
Guaiacol	0.00	0.00	C	23.92	2.05	B	60.40	2.70	A
p-ethylguaiacol	145.98	24.53	A	81.06	3.56	B	0.00	0.00	C
KETONES									
5-Valerolactone	35.97	6.97	A	22.64	1.23	B	33.31	1.66	A
g-Nonalactone	18.09	0.52	A	14.35	1.18	B	16.28	1.15	AB
TERPENES									
Limonene	22.20	1.40	B	25.23	2.32	AB	26.88	0.16	A
Nerol	6.55	2.37	A	0.00	0.00	B	0.00	0.00	B
Geranyl acetone	12.52	0.73	A	11.34	0.22	A	12.33	0.96	A

Table 6. Minor volatile compounds detected in the samples of the acetification of the craft beer (B). The letter shift throughout the process indicates significant differences at 95% confidence based on the analysis of variance (ANOVA) performed. B.S, substrate; B.EL, at the end of the loading phase; B.UL, just before the unloading phase. **Source**: own work.

VOLATILE COMPOUNDS (µg/L)	B.S			B.EL			B.UL		
	$\bar{x}$	σ		$\bar{x}$	σ		$\bar{x}$	σ	
ACIDS									
Pentanoic acid	952.47	78.31	C	5408.13	177.61	B	8562.40	523.33	A
Hexanoic acid	1845.26	74.46	A	946.27	37.53	C	1214.20	80.22	B
Octanoic acid	13268.49	423.32	A	4717.10	294.47	B	2065.95	150.26	C
Decanoic acid	1018.86	62.28	A	172.74	9.23	B	136.48	9.48	B
Dodecanoic acid	89.19	3.34	A	82.43	5.73	A	61.68	1.10	B
Tetradecanoic acid	221.34	6.97	A	212.27	16.54	A	160.07	10.33	B
Hexadecanoic acid	2241.60	46.76	A	1123.54	45.78	B	902.19	25.86	C
Octadecanoic acid	41.74	3.27	B	20.72	1.95	C	50.74	3.78	A
ALCOHOLS									
Isoamyl alcohols	178.00	4.79	A	33.88	2.52	B	14.11	1.38	C
2, 3-Butanediol	583.54	30.95	A	127.80	8.78	B	0.00	0.00	C
Furfuryl alcohol	1562.92	110.94	A	2081.34	217.65	A	1681.23	146.97	A
2-Phenylethanol	103.12	6.96	A	39.13	4.02	B	43.41	3.79	B
4-Vinylphenol	866.54	70.96	A	380.26	32.90	C	398.72	4.75	B
2-Methoxy-4-vinylphenol	1917.00	64.20	A	680.80	86.95	B	626.60	6.94	C
ALDEHYDES									
Benzaldehyde	35.71	1.94	C	246.15	5.77	B	558.15	36.04	A
Phenylacetaldehyde	163.69	16.09	A	57.10	3.63	B	50.86	3.43	B
Octanal	3.76	0.30	A	0.00	0.00	B	0.00	0.00	B
Decanal	1.75	0.09	B	2.80	0.14	A	1.82	0.07	B
3,5-Dimethylbenzaldehyde	442.26	30.67	A	185.94	23.14	B	404.02	15.76	A
KETONES									
6-Methyl-5-hepten-2-one	11.72	1.12	B	13.76	1.12	B	23.09	1.75	A
ESTERS									
Ethyl acetate	11.26	0.42	C	53.02	4.13	A	32.15	3.12	B
Isobutyl acetate	48.56	2.06	C	133.92	8.17	B	256.28	13.31	A
2-phenylethanol acetate	2548.63	171.90	B	1522.29	68.60	C	3309.62	223.58	A
Ethyl propanoate	119.86	8.66	A	104.16	5.77	B	81.85	7.85	C
Ethyl isobutyrate	1.32	0.12	C	3.83	0.13	A	2.60	0.12	B
Ethyl butanoate	684.36	26.34	A	82.26	1.87	B	32.54	1.04	C

VOLATILE COMPOUNDS (µg/L)	B.S			B.EL			B.UL		
	$\bar{x}$	σ		$\bar{x}$	σ		$\bar{x}$	σ	
ESTERS (continued)									
Ethyl pentanoate	59.58	3.06	A	0.00	0.00	B	0.00	0.00	B
Ethyl 2-methyl-2-butenoate	8.52	0.06	A	0.00	0.00	B	0.00	0.00	B
Ethyl hexanoate	728.59	12.68	A	0.00	0.00	B	0.00	0.00	B
Ethyl Cis-4-hexenoate	190.01	3.89	A	9.76	0.68	B	0.00	0.00	C
4-OH-ethyl butanoate	31.81	1.85	A	9.09	0.30	B	7.05	0.44	C
Ethyl benzoate	265.45	23.62	A	60.90	3.52	B	42.05	2.64	B
Diethyl succinate	361.31	5.84	A	316.35	17.66	B	302.90	21.21	B
Ethyl octanoate	253.98	2.62	A	3.19	0.25	B	3.22	0.21	B
Ethyl phenyl acetate	336.88	24.55	B	508.87	42.11	A	463.35	34.48	A
Ethyl phenylpropanoate	330.64	14.84	A	52.33	2.63	B	33.30	2.14	C
Ethyl isopentenyl succinate	26.84	1.48	A	28.79	2.30	A	7.95	0.55	B
2-OH-3-ethyl phenylpropanoate	26.45	4.15	A	0.00	0.00	C	13.28	0.79	B
Methyl trans-dihydrojasmonate	10.57	0.83	A	10.78	0.91	A	8.73	0.76	B
PHENOLS									
Guaiacol	0.00	0.00	C	84.08	2.84	B	129.54	5.05	A
p-ethylguaiacol	229.05	13.76	A	0.00	0.00	C	92.81	8.63	B
LACTONES									
5-Valerolactone	23.29	0.60	B	41.10	4.08	A	42.92	2.65	A
g-Nonalactone	211.21	7.72	A	169.54	10.81	B	161.18	9.97	B
TERPENES									
Limonene	23.35	0.09	A	21.65	1.27	AB	20.14	1.40	B
Nerol	20.80	1.04	A	0.00	0.00	B	0.00	0.00	B
Geranyl acetone	13.60	1.17	A	11.79	0.78	B	12.95	0.64	AB
OTHERS									
2,5,5-Trimethyl-2,6-heptadiene	279.05	1.11	A	0.00	0.00	B	0.00	0.00	B

In general, the acetification samples of the synthetic alcohol-based medium (AW) were characterized by a lower presence and content of minor volatile compounds, particularly in the raw material (AW.S). The composition of the raw material was largely distilled ethanol which was subsequently diluted in distilled water just starting the acetification. Throughout the distillation of ethanol, most of the higher alcohols, acids, esters, and aldehydes are removed, which may explain this fact. Some of these volatile compounds appeared during acetification and at the end of the process (AW.EL, AW.UL), which might be attributed to the enzymatic activity of the acetic acid bacteria (AAB), as they can oxidize several components present in the medium to organic acids, thus enhancing the metabolomic profile of vinegars (Callejón et al., 2008; Mamlouk and Gullo, 2013; Pinu et al., 2016; Sriherfyna et al., 2021).

The acetification samples of natural raw materials (FW, B) showed a higher presence, diversity, and content of volatile compounds. After a previous alcoholic fermentation of the sugars within the raw materials, a decrease in the presence and content of alcohols,

consumed by AAB, with a concomitant increase in acids presence and content could be expected throughout acetification. FW samples showed no general loss of alcohols but a significant increase in acids content at the final moments of acetification (FW.UL). For B samples, the highest richness of volatile compounds was found in the raw material (B.S). Despite significant losses in the concentration of several volatile compounds, most of them may be transferred from the substrate to the final product which, although maintaining its particularities, may acquire unique organoleptic properties from these raw materials (Palacios et al., 2002; Grierson, 2009; Ubeda et al., 2011).

These findings evidenced the need for further studies deepening the interrelationships, in this case, between the microbiota responsible for the process and differential volatile compounds to get a better understanding of the behavior of these complex systems.

3.3.3.2. Cluster analysis by Ward's method

The cluster analysis method aims at minimizing heterogeneity by assigning profiles to the clusters thereby facilitating the understanding of the relationship between them (Majerova and Nevima, 2017). Ward's method was used to discriminate among the clustering levels of vinegar samples. The presence of volatile compounds, determined by stir bar sorptive extraction (SBSE) coupled to GC-MS (see section 3.3.2.), was used as a classifying variable, thereby grouping samples based on their similarity (Figure 11).

For clustering of the samples from the three acetification processes (AW, FW, B), four different clusters can be observed, see Figure 11A. The first two clusters corresponded to the samples from the acetification of AW (AW.S, AW.EL, AW.UL) and FW (FW.S, FW.EL, FW.UL). Within them, the AW.UL and FW.S samples established subgroups differentiating them from the rest of their profile. The third cluster comprised the B.EL and B.UL samples, quite different from those of the fourth cluster, which comprised the B.S samples. Indeed, it can be appreciated that the third cluster (B.EL, B.UL) shared more features with the AW and FW samples than with those of its raw material (B.S), showing that this sampling time was particularly diverse in terms of the presence of volatile compounds. Moreover, it can be observed that samples of fine wine vinegar (FW.UL) and beer vinegar (B.UL) shared more features with each other than with those of alcohol wine vinegar (AW.UL), evidencing a higher similarity among the volatilome of the natural vinegars, see Figure 11B.

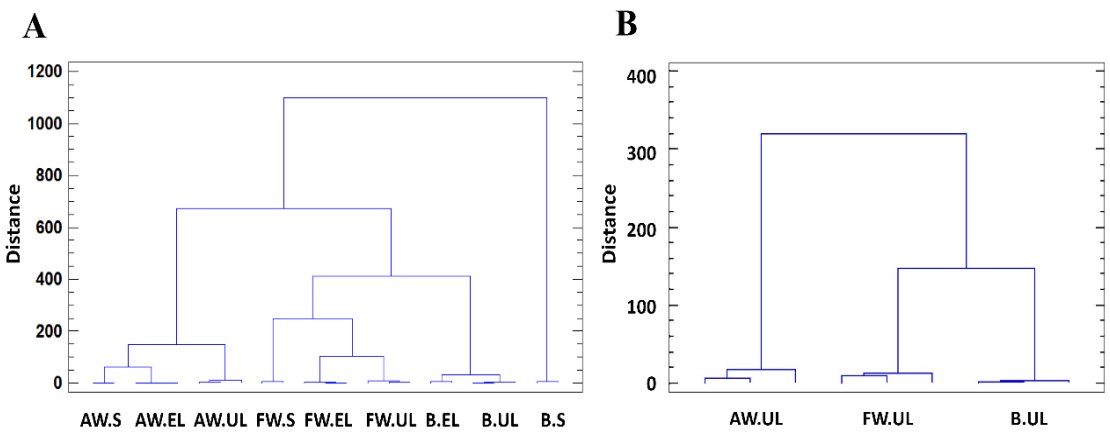

Figure 11. Cluster analysis by Ward's method of all the samples from **(A)** the complete three acetification processes (AW, FW, B) and **(B)** the three final products (AW.UL, FW.UL, B.UL). AW, synthetic alcohol-based medium; FW, fine wine; B, craft beer; S, substrate; EL, at the end of the loading phase; UL, just before the unloading phase. **Source**: own elaboration.

3.3.3.3. Principal component analysis (PCA)

Principal component analysis (PCA) is used to synthesize information by reducing the number of variables. Original variables are combined resulting in other new factors or principal components (Wold et al., 1987). Here, PCA was carried out using determined minor volatile compounds as the variable. Three principal components were identified explaining, as a whole, 82.98% of the variability of the observed data (Figure 12).

Figure 12A shows the bidimensional distribution of the selected component 1 (42.67% of the total variance) along with component 2 (22.55% of the total variance). These components displayed differences in the B.S samples against those of B.EL and B.UL. Differential variables were: 4-OH-ethyl butanoate, phenylacetaldehyde, 4-vinylphenol, ethyl phenylpropanoate, ethyl benzoate, and 2-methoxy-4-vinylphenol for component 1; and ethyl isobutyrate, 4-OH-ethyl hexanoate, 2-OH-3-ethyl phenylpropanoate, diethyl succinate, and ethyl propanoate for component 2. Figure 12B shows the bidimensional distribution of the selected component 1 (42.67% of the total variance) and component 3 (17.76% of the total variance). Component 3 presented differences in the FW.S samples against those of FW.EL and FW.UL, as well in the B.S samples against those of B.EL and B.UL. Differential variables for component 3 were guaiacol, 6-methyl-5-hepten-2-one, isobutyl acetate, 2-phenylethanol acetate, octadecanoic acid, and furfuryl alcohol.

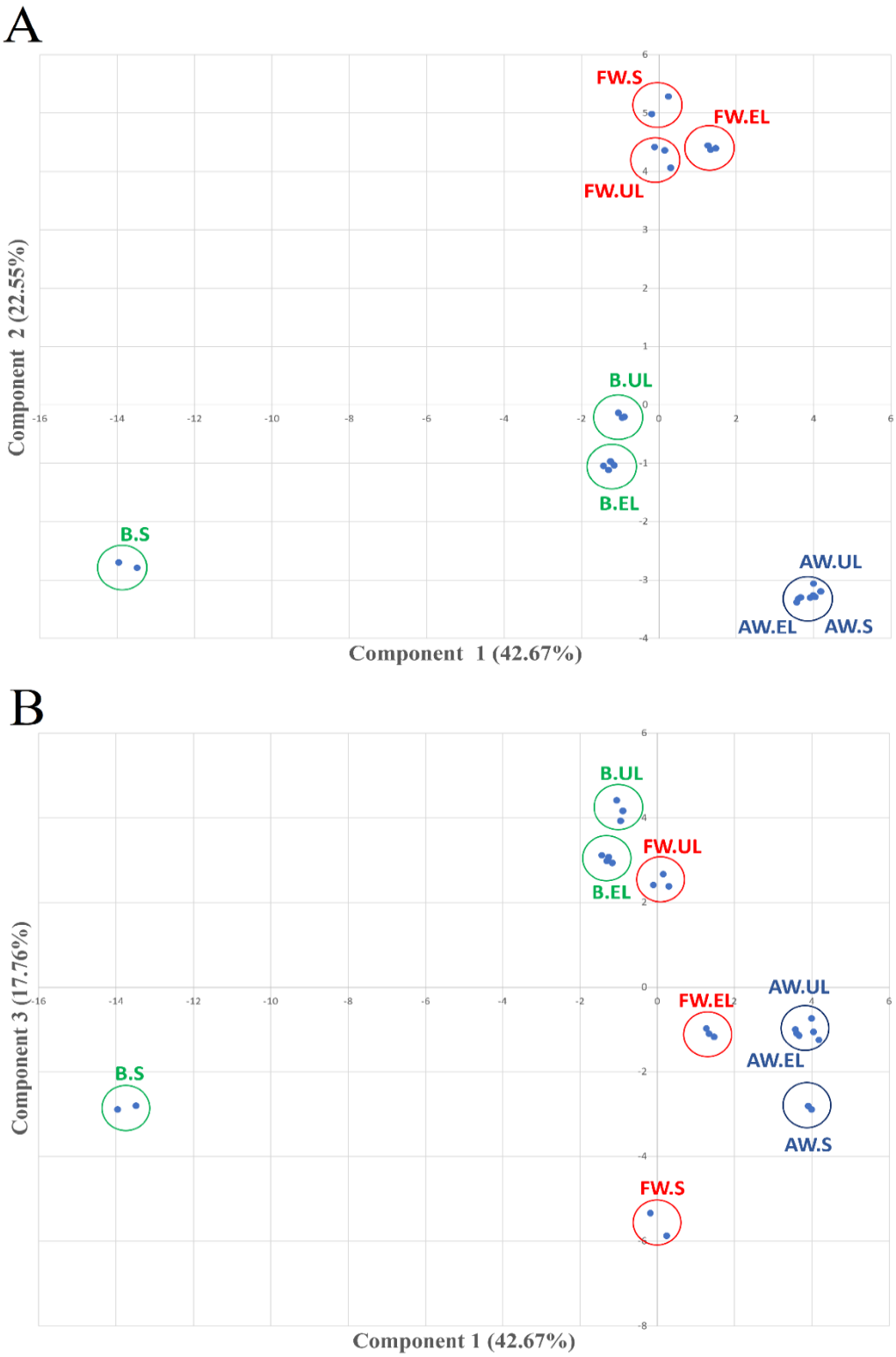

Figure 12. Principal component analysis (PCA) of all the samples from the three acetification processes (AW, FW, B) according to three components: component 1 (42.67%), component 2 (22.55%), and component 3 (17.76%). The determined minor volatile compounds were used as a variable. AW, synthetic alcohol-based medium; FW, fine wine; B, craft beer; S, substrate; EL, at the end of the loading phase; UL, just before the unloading phase. **Source**: own elaboration.

3.3.3.4. Multivariate analysis of variance (MANOVA)

The results of the MANOVA performed for the total minor volatile compounds found in all the vinegar samples showed significant differences based on three variables: the raw material or substrate used, the sampling time, and the interaction between both variables (Table 7). In general, the total minor volatile compounds were highly influenced by the raw material and sampling time, which means that the concentrations of these volatile compounds were significantly different in all the samples.

Table 7. Multivariate analysis of variance (MANOVA) performed for the total minor volatile compounds identified in all the samples of the three acetifications (AW, synthetic alcohol-based medium; FW, fine wine; B, craft beer). *, significant *p*-values < 0.05; **, significant *p*-values < 0.01; ***, significant *p*-values < 0.001; ns, non-significant values. **Source**: own work.

VOLATILE COMPOUNDS	SUBSTRATE	SAMPLING TIME	INTERACTION
ACIDS			
Pentanoic acid	***	***	***
Hexanoic acid	***	***	***
Octanoic acid	***	***	***
Decanoic acid	***	***	***
Dodecanoic acid	***	***	***
Tetradecanoic acid	***	ns	***
Hexadecanoic acid	***	***	***
Octadecanoic acid	***	***	***
ALCOHOLS			
Isoamyl alcohols	***	***	***
2, 3-Butanediol	***	***	***
Furfuryl alcohol	***	***	***
Hexanol	***	***	***
2-Phenylethanol	***	***	***
4-Vinylphenol	***	**	***
2-Methoxy-4-vinylphenol	***	***	***
ALDEHYDES			
Benzaldehyde	***	***	***
Phenylacetaldehyde	***	***	***
Octanal	***	***	***
Decanal	***	***	***
3,5-Dimethylbenzaldehyde	***	***	***
KETONES			
6-Methyl-5-hepten-2-one	***	***	***

VOLATILE COMPOUNDS	SUBSTRATE	SAMPLING TIME	INTERACTION
ESTERS			
Ethyl acetate	***	***	***
Isobutyl acetate	***	***	***
Hexyl acetate	***	***	***
2-phenylethanol acetate	***	***	***
Ethyl propanoate	***	***	***
Ethyl isobutyrate	***	***	***
Ethyl butanoate	***	***	***
Ethyl pentanoate	***	***	***
Ethyl 2-methyl-2-butenoate	***	***	***
Ethyl hexanoate	***	***	***
Ethyl Cis-4-hexenoate	***	***	***
4-OH-ethyl butanoate	***	***	***
4-OH-ethyl hexanoate	***	***	***
Ethyl benzoate	***	***	***
Diethyl succinate	***	***	***
Ethyl octanoate	***	***	***
Ethyl phenyl acetate	***	***	***
Ethyl phenylpropanoate	***	***	***
Ethyl isopentenyl succinate	***	***	***
2-OH-3-ethyl phenylpropanoate	***	***	***
Methyl trans-dihydrojasmonate	***	***	***
PHENOLS			
Guaiacol	***	***	***
p-ethylguaiacol	***	***	***
LACTONES			
5-Valerolactone	***	*	*
g-Nonalactone	***	***	***
TERPENES			
Limonene	**	ns	**
Nerol	***	***	***
Geranyl acetone	***	*	ns
OTHERS			
2,5,5-Trimethyl-2,6-heptadiene	***	***	***

3.3.4. Conclusions

The volatilome throughout the acetification processes from three different raw materials was characterized, qualitatively and quantitatively, at different moments of the process. A detailed description of the main conclusions obtained from this work can be found in section 4, just those that deal with specific Objective 3.

Section 4

CONCLUSIONS

4. CONCLUSIONS

To characterize and compare the development of three acetification profiles to study the evolution of operating conditions and the effect of diverse raw materials used (synthetic alcohol-based medium, fine wine, and craft beer) on the composition and the behavior of the microbiota responsible for the process.

The conclusions obtained from the specific Objective 1 **"*To characterize and compare the metaproteome, qualitatively and quantitatively, of the microbiota responsible for three acetification profiles (synthetic alcohol-based medium, fine wine, and craft beer) and evaluate the differential variables throughout the process*"** are divided into each of the three aspects approached in the respective publications:

I. **"*The study of the variations of system variables, as well as of the composition and main functions of the microbiota present throughout the evolution of an acetification process using a reference raw material (synthetic alcohol-based medium) through a qualitative metaproteomic approach*"** which was discussed in the work "*Metaproteomics of microbiota involved in submerged culture production of alcohol wine vinegar: A first approach*", published in the journal *"International Journal of Food Microbiology"*, allowed to draw the following conclusions:

- The acetic acid bacteria genus *Komagataeibacter* contributed to the highest fraction of the metaproteome, more than 80%, throughout the acetification of the synthetic alcohol-based medium.
- The acetic acid bacteria species *Komagataeibacter europaeus* was the predominant species of the microbiota of alcohol-based vinegar, providing almost 75% of the total proteins, followed by other closely related species of *Komagataeibacter*.
- Metaproteomics revealed a minor fraction of the microbiota composed of species from other typical genera of acetic acid bacteria (*Acetobacter*, *Gluconacetobacter*, and *Gluconobacter*) and others, not before described in industrially made vinegar.
- GO Term enrichment analysis highlighted the presence of proteins involved in catalytic activity and binding, as well as some metabolic and biosynthesis processes throughout the acetification of synthetic alcohol-based medium.

II. **"*The analysis of the quantitative differences of the metaproteome, especially on the particular proteome of the predominant microbiota, and its interactions with the non-abundant community throughout the acetification profile of synthetic alcohol medium*"** which was approached in the work "*Functional metaproteomic analysis of alcohol vinegar microbiota during an acetification process: A quantitative proteomic approach*", published in the journal "*Food Microbiology*", allowed to establish these conclusions:

- The characterization of the synthetic alcohol-based medium has allowed the establishment of a reference acetification substrate to compare with other alcoholic raw materials for making vinegar.
- The quantitative proteomic profile of the most abundant species, *K. europaeus*, allowed to predict the main aspects of the microbial community function.
- The protein activity underwent variations throughout the acetification of alcohol-based medium: the metabolism of amino acids, the biosynthesis of proteins, and energy production-related pathways prevailed during the loading phase, and then, processes related to acetic acid stress came into play at the final periods of the cycle.

III. ***"The characterization of two acetification processes from natural raw materials (fine wine and craft beer) and comparison of the influence of each one on the metaproteome of both the predominant and minor microbiota, especially, through the molecular strategies used for adaptation and survival"*** which was dealt in the work "*Unraveling the role of acetic acid bacteria comparing two acetification profiles from natural raw materials: a quantitative approach in Komagataeibacter europaeus*", published in the journal "*Frontiers in Microbiology*", led to the approach of these conclusions:

- The use of different raw materials demonstrated that neither of them barely modified the composition of the microbiota among the corresponding profiles of acetification.
- The use of a different raw material influenced the protein abundance in diverse key biological processes during the acetification process.
- The characterization of two natural acetification substrates: craft beer, a highly sugary medium, and fine wine, with a higher acetification rate, allowed suggesting a

molecular strategy of *K. europaeus* for surviving through the use of the different resources.

- The metabolization of the acetic acid, coming from ethanol oxidation, by the TCA cycle along with other energy related pathways (PPP and glycolysis), providing biosynthetic precursors, as well as membrane mechanisms for acetic acid release may be processes of biotechnological interest for the submerged vinegar production.

The conclusions obtained from the specific Objective 2 **"*Confirm the composition of the microbiota obtained through metaproteomics by applying metagenomics tools, as well as the collection and characterization of isolates from the samples taken throughout the acetification of the three working media: synthetic alcohol-based medium, fine wine, and craft beer*"** are compiled in the article "*Combining omics tools for the characterization of the microbiota of diverse vinegars obtained by submerged culture: 16S rRNA Amplicon Sequencing and MALDI-TOF MS*", published in the journal "*Frontiers in Microbiology*":

- The combination of different omics tools may be suitable for the more accurate identification of microorganisms, allowing for the achievement of a broader picture of the production of vinegar.
- Metagenomics revealed that the starter inoculum presented a higher diversity that the samples from the acetification processes.
- *Komagataeibacter* was confirmed as the main genus throughout the production of diverse vinegars by submerged culture both by metagenomics and metaproteomics.
- This omics approach allowed for the identification of taxonomic groups never to date found in vinegar made by submerged culture, highlighting the presence of archaea.

The conclusions achieved from the specific Objective 3 **"*Characterization of the "volatilome" and differentiation of the key volatile compounds throughout the evolution of the acetification of the three raw materials: synthetic alcohol-based medium, fine wine, and craft beer*"** were as follows:

- The acetification profiles obtained from the natural raw materials provided more volatile compounds than that of the synthetic alcohol medium, both qualitatively and quantitatively.
- Although the acetification profiles from the natural raw materials showed a similar number of volatile compounds, the volatilome composition differed among them in qualitative and quantitative terms.
- Craft beer was a raw material particularly rich in minor volatile compounds.
- The cluster analysis by Ward's method revealed that the volatilome from the acetification profiles of fine wine and craft beer shared more features among them than with that of the synthetic alcohol medium, especially at the final of the process.
- Principal component analysis (PCA) allowed for obtaining three unique components explaining, in their combination, around 83% of the observed variability. A total of 17 minor volatile compounds were the main variables of the system.

General Conclusions and Future Perspectives

The research conducted in this Doctoral Thesis has allowed for characterizing diverse acetification profiles using several alcoholic raw materials as substrates for vinegar production by submerged culture. The use of different omics tools allowed to describe and confirm the composition of these microbiota, mainly comprised of the AAB genus *Komagataeibacter*, with the species *K. europaeus* as the main representative, and a less-abundant microbiota composed of closely related species, other species from typical AAB genera, numerous groups of bacteria, other than AAB, and even archaea groups. Some of these groups of microorganisms have been for the first time described in vinegar obtained in the present Doctoral Thesis. From a quantitative protein approach, it has been demonstrated that the use of different raw materials barely modified the composition of the microbiota, but it did cause significant variations in the activity, behavior, and molecular strategies used throughout the acetification.

This work, which identifies the microbiota of the process as well as multiple volatile compounds, important for the sensory properties of the final products, is a preliminary, fundamental stage before being able to approach, in the most rigorous way possible, the study of the interrelationships existing in complex systems such as this one. It is providing new relevant findings that contribute both to the improvement of the existing

current knowledge on the role of the microbial community members as well as expand the use of diverse raw materials in industrial vinegar-making. In the medium-long term, these achievements might lead to the improvement of operating conditions allowing to obtain of new types of vinegar with improved organoleptic properties and high quality. The use of improved starter cultures from the selection of species or strains with a crucial role during acetification might contribute to this purpose. Progress in the field of obtaining vinegar isolates, their phenotypic characterization, and biotechnological enhancement will be crucial in this regard. Among new types of vinegar, the study of those with a high presence of gluconic acid, which may confer to the final product greater stability and an acid flavor with a mild sweetness, is currently carried out by our group. Finally, it is worth noting that, from the general omics studies performed in this Doctoral Thesis, future work focused on obtaining marker genes, proteins, and metabolites during vinegar production might lead to the use of more specific strategies for the improvement of the operating conditions.

Section 5

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5. REFERENCES

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Section 6

APPENDIX

6. APPENDIX

6.1. Scientific contributions derived from the Doctoral Thesis

6.1.1. Scientific publications

I. Román-Camacho, J. J., Santos-Dueñas, I. M., García-García, I., Moreno-García, J., García-Martínez, T., Mauricio, J. C. (2020). Metaproteomics of microbiota involved in submerged culture production of alcohol wine vinegar: A first approach. *International Journal of Food Microbiology*. 333, 108797. https://doi.org/10.1016/j.ijfoodmicro.2020.108797.
(*Chapter I*)

II. Román-Camacho, J. J., Mauricio, J. C., Santos-Dueñas, I. M., García-Martínez, T., García-García, I. (2021). Functional metaproteomic analysis of alcohol vinegar microbiota during an acetification process: A quantitative proteomic approach. *Food Microbiology*. 98, 103799. https://doi.org/10.1016/j.fm.2021.103799.
(*Chapter II*)

III. Román-Camacho, J. J., Mauricio, J. C., Santos-Dueñas, I. M., García-Martínez, T., García-García, I. (2022). Unraveling the role of acetic acid bacteria comparing two acetification profiles from natural raw materials: a quantitative approach in *Komagataeibacter europaeus*. *Frontiers in Microbiology*. 13, 840119. https://doi.org/10.3389/fmicb.2022.840119.
(*Chapter III*)

IV. Román-Camacho, J. J., García-García, I., Santos-Dueñas, I. M., Ehrenreich, A., Liebl, W., García-Martínez, T., Mauricio, J. C. (2022). Combining omics tools for the characterization of the microbiota of diverse vinegars obtained by submerged culture: 16S rRNA Amplicon Sequencing and MALDI-TOF MS. *Frontier in Microbiology*. **ACCEPTED**.
(*Chapter IV*)

6.1.2. Congress communications

I. Román-Camacho, J. J. (2019). Mejora de la elaboración de vinagre a través del estudio integrado del microbioma y metaboloma. "*I Jornada de Transferencia*

Universidad-Empresa del Máster en Biotecnología. Levaduras y bacterias acéticas: ejemplos de éxito biotecnológico". Universidad de Córdoba (Córdoba). Oral communication.

II. Román-Camacho, J. J., Santos-Dueñas, I. M., García-García, I., Mauricio, J. C. (2020). Metaproteómica cualitativa de la microbiota de vinagre de alcohol. "*VIII Congreso Científico de Investigadores en Formación"*. Universidad de Córdoba (Córdoba). Poster communication.

III. Román-Camacho, J. J., Mauricio, J. C., García-García, I., Santos-Dueñas, I. M., García-Martínez, T. (2021). Perfil metaproteómico de la microbiota de vinagre de alcohol vínico durante un proceso de acetificación en modo semicontinuo: composición y análisis funcional. "*Jornadas Científicas de GIENOL*". Universidad de Murcia (Murcia). Oral communication.

IV. Román-Camacho, J. J., García-García, J. C., Santos-Dueñas, I. M., García-Martínez, T., Mauricio, J. C., García-García, I. (2021). Aplicación de técnicas multi-ómicas para la identificación de la microbiota del vinagre. "*III Congreso de Veterinaria y Ciencia y Tecnología de los Alimentos*". Universidad de Córdoba (Córdoba). Poster communication.

V. Román-Camacho, J. J., Santos-Dueñas, I. M., García-Martínez, T., García-García, I., Mauricio, J. C. (2021). Metaproteómica de bacterias acéticas durante la acetificación de vino y cerveza. "*XXVIII Congreso de la Sociedad Española de Microbiología*" (Virtual Congress). Poster communication.

VI. Román-Camacho, J. J., Mauricio, J. C., Santos-Dueñas, I. M., García-Martínez, T., García-García, I. (2022). Análisis de proteínas de bacterias acéticas en dos sustratos: vino fino y cerveza artesana. "*XV Congreso Nacional de Investigación Enológica. GIENOL*". Universidad de Murcia (Murcia). Chapter of a book of communications.

VII. Mauricio, J. C., Román-Camacho, J. J., Santos-Dueñas, I. M., García-Martínez, T., García-García, I. (2022). Análisis de proteínas de bacterias acéticas en dos sustratos: vino fino y cerveza artesana. "*XV Congreso Nacional de Investigación Enológica. GIENOL*". Universidad de Murcia (Murcia). Poster communication.

VIII. Mauricio, J. C., Carbonero-Pacheco, J. R., Alcalá-Jiménez, M. T., Román-Camacho, J. J., García-García, J. C., Moreno-García, J., Moreno, J., Santos-Dueñas, I. M., García-García, I., García-Martínez, T. (2022). Biodiversidad de levaduras en mostos y vinos de la DOP Montilla-Moriles. "*XV Congreso Nacional de Micología*". Universitat de València (Valencia). Poster communication.

6.2. Scientific contributions not derived from the Doctoral Thesis

6.2.1. Scientific publications

I. Porras-Agüera, J. A., Román-Camacho, J. J., Moreno-García, J., Mauricio, J. C., Moreno, J., García-Martínez, T. (2020). Effect of endogenous CO_2 overpressure on the yeast "stressome" during the "prise de mousse" of sparkling wine. *Food microbiology*. 89, 103431. https://doi.org/10.1016/j.fm.2020.103431.

6.2.2. Congress communications

I. Román-Camacho, J. J., Porras-Agüera, J. A., Moreno-García, J., Mauricio, J. C., Moreno, J., García-Martínez, T. (2018). Efecto de la presión de CO_2 endógeno sobre la expresión de proteínas de estrés de *Saccharomyces cerevisiae* durante la segunda fermentación del cava. "*VII Congreso Nacional de Microbiología Industrial y Biotecnología Microbiana*". Universidad de Cádiz (Cádiz). Poster communication.

II. Santos-Dueñas, I. M., Cañete-Rodríguez, A. M., Román-Camacho, J. J., García-Martínez, T., Mauricio, J. C., García-García, I. (2019). Influencia de la composición del medio en la producción de ácido glucónico empleando *Gluconobacter oxydans*. "*XXXVI Jornadas Nacionales de Ingeniería Química*". Universidad de Zaragoza (Zaragoza). Poster communication.

III. Santos-Dueñas, I. M., Cañete-Rodríguez, A. M., Román-Camacho, J. J., García-Martínez, T., Mauricio, J. C., García-García, I. (2019). Preparación y conservación de inóculos para la acetificación de vinagre de vino. "*XXXVI Jornadas Nacionales de Ingeniería Química*". Universidad de Zaragoza (Zaragoza). Poster communication.

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