11-2023

CHARACTERIZATION OF THE PROBIOTIC PROPERTIES OF YEAST STRAINS ISOLATED FROM DIFFERENT FOOD SOURCES AND UNRAVELING THE POTENTIAL NUTRITIONAL BENEFITS OF FERMENTED DATE PALM POMACE WITH SELECTED PROBIOTIC CANDIDATES: UNTARGETED METABOLOMICS AND CARBOHYDRATE METABOLITES OF IN VITRO DIGESTED FRACTION

Nadia Al Kalbani
United Arab Emirates University, 950223010@uaeu.ac.ae

UAEU
جامعة الإمارات العربية المتحدة
United Arab Emirates University

DOCTORATE DISSERTATION NO. 2023: 47

College of Agriculture and Veterinary Medicine

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Nadia Al Kalbani

This dissertation is submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Food Science and Technology

November 2023

United Arab Emirates University Doctorate Dissertation
2023: 47

Cover: Exopolysaccharides produced by *P. kudriavzevii*

(Photo: By Nadia Al Kalbani)

Print: University Print Service, UAEU 2023

Declaration of Original Work

I, Nadia Al Kalbani, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this dissertation entitled "*Characterization of the Probiotic Properties of Yeast Strains Isolated from Different Food Sources and Unraveling the Potential Nutritional Benefits of Fermented Date Palm Pomace with Selected Probiotic Candidates: Untargeted Metabolomics and Carbohydrate Metabolites of In Vitro Digested Fraction*.", hereby, solemnly declare this is the original research work done by me under the supervision of Dr. Mutamed Ayyash, in the College of Agriculture and Veterinary Medicine at UAEU. This work has not previously formed the basis for the award of any academic degree, diploma or a similar title at this or any other university. Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my dissertation have been properly cited and acknowledged in accordance with appropriate academic conventions. I further declare that there is no potential conflict of interest with respect to the research, data collection, authorship, presentation and/or publication of this dissertation.

Student's Signature: 

Date: 14/11/2023

Advisory Committee

1) Advisor: Dr. Mutamed Ayyash

Title: Associate Professor

Department of Food Science

College of Agriculture and Veterinary Medicine

2) Co-advisor: Prof. Afaf Kamal-Eldin

Title: Professor

Department of Food Science

College of Agriculture and Veterinary Medicine

3) Member: Prof. Khaled A. El-Tarabily

Title: Professor

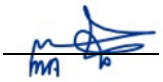
Department of Biology

College of Science

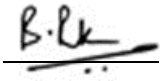
Approval of the Doctorate Dissertation

This Doctorate Dissertation is approved by the following Examining Committee Members:

1) Advisor (Committee Chair): Dr. Mutamed Ayyash
   Title: Associate Professor
   Department of Food Science
   College of Agriculture and Veterinary Medicine

   Signature  Date 14/11/2023

2) Member: Dr. Raman Bhaskaracharya
   Title: Assistant Professor
   Department of Food Science
   College of Agriculture and Veterinary Medicine

   Signature  Date 14/11/2023

3) Member: Prof. Basim Abu-Jdayil
   Title: Professor
   Department of Chemical and Petroleum Engineering
   College of Engineering

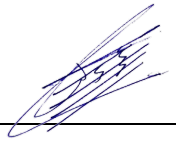
   Signature  Date 14/11/2023

4) Member (External Examiner): Prof. Huang Deijan
   Title: Professor
   Department of Food Science and Technology
   Institution: The National University of Singapore, Singapore

   Signature  Date 14/11/2023

This Doctorate Dissertation is accepted by:

Acting Dean of the College of Agriculture and Veterinary Medicine: Dr. Mohammad Alyafei

Signature 

Date 18/12/2023

Dean of the College of Graduate Studies: Professor Ali Al-Marzouqi

Signature 

Date 18/12/2023

Abstract

The use of agricultural by-products, including date palm pomace (DPP), presents a promising opportunity for advancing sustainable food production. By fermenting DPP with novel probiotic yeast candidates, the bioactive properties of the by-product can be enhanced, potentially yielding health benefits. This dissertation aimed to (1) isolate yeast from fermented food products as well as to characterize their probiotic properties; (2) investigate the physiochemical properties of fermented DPP by selected yeast isolates; (3) evaluate the health benefits of non-fermented and fermented DPP samples both pre and post-digestion (bioaccessible portions); and (4) analyze the untargeted metabolites and carbohydrates metabolites in the bioaccessible portions. The yeast isolates showed remarkable survivability in acidic environment with a reduction of 0.7 to 2.1 Log_{10} under *in vitro* digestion conditions. Bile salt tolerance increased with incubation time, ranging from 69.2% to 91.1% after 24 hours. Out of 105 isolates obtained, the 12 promising isolates were selected for further screening of their probiotic characteristics. These isolates exhibited a wide range of cholesterol removal abilities, varying from 41.6% to 96.5%. All 12 yeast strains had good cell surface properties and showed the capability to hydrolyze investigated bile salts display heat resistance, possess potent antimicrobial activity, and engage in EPS production. The ability to adhere to the HT-29 cell line was an average of 6.3 Log_{10} CFU/mL after 2 h. The study identified 12 yeast isolates through ITS/5.8S ribosomal DNA sequencing, consisting of one strain each of *C. albicans, S. cerevisiae*, *I. orientalis* and *P. cecembensis*, and eight strains of *P. kudriavzevii*. Four distinctive isolates, namely *P. cecembensis*, *I. orientalis*, *S. cerevisiae*, and *P. kudriavzevii*, were selected for fermenting DPP. The fermentation of DPP resulted in the abundance of malic acid (over 2400 mg/100g) and the identification of 42 volatiles, with different predominance observed in the samples. Twenty phenols were determined by U-HPLC in fermented DPP, with (-)-epicatechin being the most abundant. Bioaccessibility studies revealed that fermented DPP samples exhibited improved α-amylase inhibition compared to non-fermented and undigested samples. *In vitro* cytotoxicity assays showed a more potent inhibitory effect of fermented DPP against MCF-7 and Caco2 cell lines (average inhibition of 55.7% and 74.9%, respectively) compared to non-fermented DPP (27.8% and 33.2%). Two yeast isolates, *I. orientalis and P. kudriavzevii*, were used to

analyze untargeted metabolites and carbohydrate metabolites. The untargeted metabolomics analysis identified C5-branched dibasic acid metabolism and biosynthesis of secondary metabolites as the most prominent pathways in fermented samples by *P. kudriavzevii and I. orientalis, respectively*. Moreover, The LC-QTOF analysis of bioaccessible carbohydrate metabolites revealed the presence of two phytochemical groups, alkaloids and terpenoids, in the *I. orientalis* sample and three terpenoid metabolites in the *P. kudriavzevii* sample. The dissertation emphasizes the feasibility of utilizing probiotic yeast candidates to convert fermented food waste, such as DPP, into a sustainable source of bioactive compounds. This research provides an understanding of the physiochemical properties and metabolic pathways involved in fermented DPP, contributing to the development of functional foods.

Keywords: Date pomace, antioxidant, antidiabetics, cytotoxicity, phenolic compounds, untargeted metabolites.

Title and Abstract (in Arabic)

توصيف خصائص البروبيوتيك لسلالات الخميرة المعزولة من مصادر غذائية مختلفة وكشف الفوائد الغذائية المحتملة لثفل نخيل التمر المخمر: المستقلبات غير المستهدفة ومستقلبات الكربوهيدرات في الجزء المهضوم مخبريًا

الملخص

يُعد استخدام المنتجات الثانوية الزراعية، بما في ذلك ثفل التمور، فرصة واعدة لتعزيز الإنتاج الغذائي المُستدام. يمكن تحسين الخصائص النشطة بيولوجيًا لثفل التمور من خلال تخميرها باستخدام فطريات تحمل خصائص البروبيوتيك، الأمر الذي قد يدعم الفوائد الصحية المحتملة لثفل التمور. هدفت هذه الرسالة إلى (1) عزل الفطر من المنتجات الغذائية المُخمرة وتوصيف خصائصها كبروبيوتيك مُحتملة. (2) دراسة الخصائص الكيميائية لـثفل التمور المُخمرة بواسطة عزلات فطر مختارة؛ (3) تقييم الفوائد الصحية لعينات ثفل التمور غير المُخمرة والمُخمرة قبل وبعد الهضم مخبريًا؛ (4) وتحليل المستقلبات غير المستهدفة ومستقلبات الكربوهيدرات في الأجزاء التي تم هضمها مخبريًا. أظهرتْ عزلات الفطر قابلية بقاء ملحوظة في البيئة الحمضية مع انخفاض من Log_{10} 0.7 - 2.1 تحت ظروف الهضم في المختبر. زاد تحمل الأملاح الصفراوية مع فترة الحضانة، حيث تراوح من 69.2٪ إلى 91.1٪ بعد 24 ساعة. من بين 105 عزلة تم تقييم تحملها لظروف الهضم مخبريًا، اختيرتْ 12 عزلة واعدة، لمزيد من الفحوصات لخصائصها ككائنات دقيقة تمتلك خصائص البروبيوتيك. تمتعتْ العزلات الـ 12 المُختارة بقدرة على إزالة الكوليسترول تراوحت من 41.6٪ إلى 96.5٪. وامتلكت العزلات المُختارة خصائص جيدة لسطح الخلية، كما أظهرت القُدرة على التحلل المائي للأملاح الصفراوية التي تعرضتْ لها، وكان لها أيضًا القدرة على مقاومة الحرارة، ونشاط قوي لمضادات الميكروبات، إضافة لذلك، امتلكت جميع العزلات المُختارة القدرة على إنتاج البولي سكاريد الخارجي. بينما قُدِّرتْ كفاءة عزلات الفطر بعد ساعتين من الالتصاق بخط خلية HT-29 بمتوسط 6.3 Log_{10} CFU / mL. تم تعريف الاثنتي عشر عزلة من خلال تسلسل الحمض النووي الريبوزومي ITS/5.8S كالتالي: سلالة واحدة لكلٍ من *C. albicans*، *S. cerevisiae*، *I. orientalis*، و*P. cecembensis*، وثمان سلالات *P kudriavzevii*. تم اختيار أربع عزلات مميزة وهي *P. cecembensis* و *I. orienta*lis و *S. cerevisiae* و *P. kudriavzevii* لتخمير ثفل التمور. أدى تخمر ثفل التمور إلى وفرة حمض الماليك (أكثر من 2400 مجم / 100 جم) وتحديد 42 مادة متطايرة، مع ملاحظة هيمنة متفاوتة من المواد المتطايرة بين الأربع عينات. تم تحديد عشرين فينول بواسطة U-HPLC في ثفل التمور المخمر، حيث كان فينول (epicatechin(-)-) هو الأكثر وفرة. كشف اختبار الأجزاء المهضومة في المختبر أن عينات ثفل التمر المخمرة حسّنت تثبيط ألفا-أميليز مقارنة بالعينات غير المخمرة وغير المهضومة. إضافة لذلك، أظهرت فحوصات السميَّة الخلوية في المختبر أن تأثير ثفل التمور المخمرة ضد MCF-7 وCaco2 كان أقوى (بمتوسط تثبيط 55.7٪ و74.9٪ على التوالي) مقارنة بـثفل التمور الغير مخمرة (27.8٪ و33.2٪). تم استخدام عزلتين وهما، *I. orienta*lis و *P. kudriavzevii*، لتحليل المستقلبات غير المستهدفة ومستقلبات الكربوهيدرات. حدد تحليل الأيض غير المستهدف كل من C5-branched

dibasic and metabolism و biosynthesis of secondary metabolites كأبرز المسارات في العينات المخمرة بواسطة *P. kudriavzevii* و*I.orientalis*، على التوالي. علاوة على ذلك، أظهر تحليل LC-QTOF لمستقلبات الكربوهيدرات المهضومة مخبريًا وجود مجموعتين من المركبات النباتية؛ وهُما القلويدات والتربينويدات في عينة *I. orientalis*، وثلاثة مستقلبات تربينويد في عينة *P. kudriavzevii*. تؤكد الأطروحة على جدوى استخدام الكائنات الحية المجهرية كالفطريات المرشحة لامتلاك خصائص البروبيوتيك لتحويل نفايات الطعام المخمرة، مثل ثفل التمور، إلى مصدر مستدام للمركبات النشطة بيولوجيًا. يقدم هذا البحث شرحًا للخصائص الكيميائية والمسارات الأيضية في ثفل التمور المخمرة، مما يقد يساهم في تطوير الأطعمة الوظيفية.

مفاهيم البحث الرئيسية: ثفل التمر، مضادات الأكسدة، مضادات السكر، السمية الخلوية، الفينولات، المستقلبات الغير مستهدفة.

List of Publications

This dissertation is based on the work presented in the following papers, referred to by Roman numerals.

I. Alkalbani, N. S., Osaili, T. M., Al-Nabulsi, A. A., Olaimat, A. N., Liu, S. Q., Shah, N. P., Apostolopoulos, V., & Ayyash, M. M. (2022). Assessment of Yeasts as Potential Probiotics: A Review of Gastrointestinal Tract Conditions and Investigation Methods. Journal of Fungi, 8(4). doi:10.3390/jof8040365

II. Alkalbani, N. S., Osaili, T. M., Al-Nabulsi, A. A., Obaid, R. S., Olaimat, A. N., Liu, S. Q., & Ayyash, M. M. (2022). In Vitro Characterization and Identification of Potential Probiotic Yeasts Isolated from Fermented Dairy and Non-Dairy Food Products. J Fungi (Basel), 8(5), 544. doi:10.3390/jof8050544

III. Alkalbani, N. S., Alam, M. Z., Al-Nabulsi, A., Osaili, T. M., Olaimat, A., Liu, S. Q., Kamal-Eldin, A., & Ayyash, M. (2023). Fermentation of Date Pulp Residues Using Saccharomyces cerevisiae and Pichia kudriavzevii—Insights into Biological Activities, Phenolic and Volatile Compounds, Untargeted Metabolomics, and Carbohydrate Analysis Post In Vitro Digestion. Fermentation, 9(6). doi:10.3390/fermentation9060561

IV. Alkalbani, N.S.; Alam, M.Z.; Al-Nabulsi, A.; Osaili, T.M.; Obaid, R.R.; Liu, S.-Q.; Kamal-Eldin, A., & Ayyash, M. Unraveling the Potential Nutritional Benefits of Fermented Date Syrup Waste: Untargeted Metabolomics and Carbohydrate Metabolites of In Vitro Digested Fraction. Food Chemistry*, (Manuscript Submitted and Accepted).*

Author's Contribution

The contribution of Nadia Al Kalbani to the papers included in this dissertation was as follows:

I. Contributed to the planning of the research, took primary responsibility for collecting and curating data, analysis of the results, and manuscript writing.

II. Contributed to the planning of the research, had major responsibility for conducting almost all experimental work, collecting and curating data, preparation of the tables and figures, analysis of the results, and manuscript writing.

III. Contributed to the planning of the research, had major responsibility for conducting almost all experimental work, collecting and curating data, preparation of the tables and figures, analysis of the results, and manuscript writing.

IV. Contributed to the planning of the research, had the main responsibility for conducting almost all experimental work, collecting and curating data, preparation of the tables and figures, analysis of the results, and manuscript writing.

Acknowledgements

It gives me great pleasure to acknowledge the significant roles played by several individuals who were pivotal in the completion of my PhD research.

I wish to extend my heartfelt gratitude to the United Arab Emirates University and the College of Graduate Studies for providing me with the invaluable opportunity to pursue and attain this PhD degree. I am profoundly thankful to my supervisor, Dr. Mutamed Ayyash, for his steadfast support throughout my PhD journey. His patience, motivation, and profound knowledge have been indispensable in guiding me through my research and culminating in the completion of this dissertation.

I am also indebted to my advisory committee members, Prof. Afaf Kamal Eldin and Prof. Khalid El Tarabily, for their dedicated efforts and insightful suggestions that have significantly enhanced this research.

The realization of this work would not have been feasible without the generous contributions of the faculty members and technicians in the College of Agriculture and Veterinary Medicine. Special gratitude goes to Dr. Hassan Abdullah, Mr. Usama Souka, Dr. Jaleel Kizhakkayil, Mr. Ismail, and Dr. Mohamed Enan from the Biology Department for their substantial contributions. I would also like to express my appreciation to Ms. Muneeba for her technical support.

Lastly, I reserve warm thanks for my family members, particularly my remarkable mother, for their unwavering moral support and boundless encouragement.

Dedication

To the soul of my father and the heart of my mother

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List of Abbreviations

ACE	Angiotensin-Converting Enzyme
BSH	Bile Salt Hydrolysis
CA	Cholic Acid
CDSW	Unfermented Date Syrup Waste
DNA	Deoxyribonucleic Acid
DPP	Date Palm Pomace
DPR	Date Pulp Residues
DSW	Date Syrup Waste
EPS	Exopolysaccharides
FAO	Food and Agriculture Organization
FDSW	Fermented Date Syrup Waste
GIT	Gastrointestinal Tract
KEGG	Kyoto Encyclopedia of Genes and Genomes
LAB	Lactic Acid Bacteria
LC-QTOF	Liquid Chromatography Quadrupole Time-of-Flight
MIC	Minimum-Inhibitory Concentration
NCBI	National Centre for Biotechnology Information
NIC	Non-Inhibitory Concentration
OX	Oxgall
PCA	Principal Component Analysis
PRA	Relative Peak Area
QPS	Qualified Presumption of Safety
SD	Standard Deviation
SGF	Simulated Gastric Fluid

SIF	Simulated Intestinal Fluid
SSF	Simulated Salivary Fluid
TA	Taurocholic Acid
TPC	Total Phenolic Compounds
U-HPLC	Ultra-High Performance Liquid Chromatography
UPLC-QTOF	Ultra-High Performance liquid chromatography with Quadrupole Time-of-Flight Mass Spectrometry
WHO	World Health Organization
YPD	Yeast Extract-Peptone-Dextrose

Chapter 1: Introduction

1.1 Overview

The term ‘probiotics’ refers to ‘live microorganisms that, when administered in adequate amounts, confer a health benefit on the host’ (Hill et al., 2014). Functional foods are food products that contain probiotics and provide a range of health benefits, such as antihypertensive, hypoglycemic, antioxidant (Ashaolu, 2020; Reque & Brandelli, 2021; Wang et al., 2021b), and immunomodulatory effects (Dargahi et al., 2021). Several clinical research has consistently demonstrated the beneficial impacts of probiotics, which can be consumed either as dietary supplements or incorporated into food products (Gu et al., 2014; Lee et al., 2014; Wong et al., 2015).

When creating functional food with the aim of maximizing the potential benefits of probiotics, it is crucial to select an appropriate food matrix. This matrix plays a crucial role in creating a favorable environment that supports their growth and activity (Flach et al., 2018). By doing so, it enables microbial bioconversion, which ultimately contributes to the release of bioactive compounds relevant to health-promoting benefits.

Date palm (*Phoenix dactylifera L.*) is a long-standing and economically important crop cultivated in the Middle East and North Africa, where dates are a staple food (Oladzad et al., 2021). Date palm pomace (DPP) refers to the biomass derived from the side-stream of date processing industries, specifically the residue left after the extraction of syrup or juice (Rambabu et al., 2020). Scientific research has highlighted the substantial nutritional content of date processing waste, making it a valuable raw material for the production of high-value bioproducts (Ahmad et al., 2021; Seyed Reihani & Khosravi-Darani, 2019). In this regard, microbial bioconversion, involving yeast, can be employed to utilize the dietary fiber and yeast-fermentable sugars present in DPP as a source of nutrients and carbon for producing desired value-added products (Munekata et al., 2021; Xiang et al., 2019).

1.2 Problem Statement

This dissertation attempts to address two main issues in one topic, both of which revolve around the exploitation of natural resources. Firstly, yeast cells, which are larger than bacteria, can hinder the growth of harmful bacteria, making them a potential probiotic candidate (Czerucka et al., 2007). However, only a few strains of *Saccharomyces cerevisiae* (*S. cerevisiae*) have been identified and are commercially available as probiotics for humans (Arévalo-Villena et al., 2018). Before conducting clinical trials, it is important to characterize potential probiotic yeasts *in vitro*. Therefore, it is necessary to isolate non-*S. cerevisiae* strains, alongside *S. cerevisiae*, and subsequently assess their potential as probiotic candidates.

Secondly, on the other hand, around 17-28% of DPP is produced daily by date juicing industries, with its primary usage being as animal feed or discarded in open areas (Heidarinejad et al., 2018). Date waste, which contains residual pulp and sugars, has a high moisture content and is easily fermentable, causing significant challenges in waste management (Oladzad et al., 2021). This situation also poses a risk of disease by providing a conducive environment for pests, mice, and microorganism growth. However, several studies have reported that DPP is abundant in carotenoids, dietary fiber, phenolic compounds, and minerals (Fernández-López et al., 2022; Oladzad et al., 2021; Struck & Rohm, 2020). DPP presents opportunities for extracting bioactive compounds and producing innovative bio-based products using biorefinery technologies, which is beneficial both from a nutritional and economic standpoint. To mitigate the negative environmental impacts of DPP and promote sustainability in the date fruit industry, effective strategies are necessary. The fermentation of agro-food by-products is a well-established method for valorizing such materials, as it enables the development of valuable food products and ensures food preservation in a sustainable manner (Chandrasekaran & Bahkali, 2013; Montero-Zamora et al., 2022; Sabater et al., 2020).

Considering all of this, utilizing novel yeast isolates to ferment food waste, such as DPP, has the potential to contribute to advancements in food sustainability within the principles of a green economy.

1.3 Research Objectives

The biofunctional market necessitates a continuous demand for the diversification and application of novel products that offer new probiotic strains with specific functional properties (de Melo Pereira et al., 2018). Probiotic yeasts possess functional characteristics that bacterial probiotics lack, making the isolation of new probiotic yeasts an ongoing requirement to meet the demands of the functional food and beverage market. Additionally, the scientific community has shown interest in adopting the circular bio-economy concept by leveraging the value of fruit by-products. This dissertation suggests that microbial bioconversion can be performed using the dietary fiber and yeast-fermentable sugars present in DPP as a nutrient and carbon source to produce the targeted value-added products. As a result, the objectives of this dissertation were to:

(1) Isolate novel yeasts from fermented food products.

(2) Characterize the potential probiotic attributes of these newly isolated yeasts, including tolerance to the gastrointestinal tract (GIT) conditions, cell surface and adhesive properties (autoaggregation, hydrophobicity, coaggregation and HT-29 cell line adhesion) antimicrobial activities, antibiotic sensitivities, heat tolerance, exopolysaccharides (EPS) production, ability to remove cholesterol and bile salt hydrolysis (BSH) activity.

(3) Identify the best potential probiotic yeasts using molecular techniques.

(4) Analyze the chemical characteristics of fermented DPP and non-fermented DPP by novel yeast isolates, including organic acids and sugars, volatile compounds, and phenolic compounds.

(5) Evaluate their health-promoting benefits comprising antidiabetic, antioxidant, cytotoxicity, total phenolic compounds (TPC), proteolytic activity, and (angiotensin-converting enzyme) ACE inhibition, before and after *in vitro* digestion of bioaccessible portions.

(6) Identify untargeted and carbohydrate metabolites in the bioaccessible portions using UPLC-QTOF.

1.4 Relevant Literature

1.4.1 Identification of Microorganisms as Probiotic

Yeasts, which are unicellular eukaryotic microorganisms, are commonly present in soil, air, water, and various food sources. They can be derived from both animal and plant origins and make up less than 0.1% of the microbiota in the human gut (Foligne et al., 2010). Recently, there has been growing interest in utilizing yeasts as probiotics due to their abundant mineral and vitamin B content, as well as their high levels of peptides, proteins, and several immunostimulant compounds, including mannan oligosaccharides, proteases, and β-glucans (Arévalo-Villena et al., 2018; Czerucka et al., 2007; Fadda et al., 2017; Gil-Rodríguez et al., 2015).

The characterization of novel probiotic candidates, including yeast, is required to align with the criteria set forth by the Food and Agriculture organization/World Health Organization (FAO/WHO) in 2002. Of utmost importance among these guidelines is the ability to withstand the conditions of the GIT (low pH, digestive enzymes, bile salts, and alkaline pH) (Vera-Pingitore et al., 2016). Additionally, the probiotic candidates should possess qualities such as adherence to epithelial cells, bile salt hydrolysis (BSH), assimilation of cholesterol in both the human intestine and food, antimicrobial properties, and sensitivity to antibiotics (FAO/WHO, 2002). For industrial purposes, it is essential for probiotic candidates to exhibit tolerance to high temperatures and the capacity to produce exopolysaccharides (EPS) (Silambarasan et al., 2019). Moreover,, when choosing probiotics, it is crucial to consider safety aspects such as the absence of acquired antimicrobial resistance genes or recognized virulence factors (Binda et al., 2020).

The potential mechanisms underlying probiotic action include their ability to secrete antimicrobial substances, compete with the pathogens for nutrients and adherence to the mucosa and epithelium in GIT, and modulate the immune system (Halloran & Underwood, 2019; Ng et al., 2008; Oelschlaeger, 2010).

Chapter 2 of this dissertation elaborated the adaptations and mechanisms underlying the survival of probiotic yeasts under GIT and salt stresses. This chapter also discussed the capability of yeasts to adhere to epithelial cells (hydrophobicity and

autoaggregation) and shed light on *in vitro* methods used to assess the probiotic characteristics of newly isolated yeasts. While Chapter 3 is about the isolation of yeast from fermented dairy and non-dairy products as well as the characterization of their survival in *in vitro* digestion conditions and tolerance to bile salts. Also, other probiotic properties were investigated and discussed in Chapter 3, these including cell surface properties (autoaggregation, hydrophobicity and coaggregation), physiological properties (adhesion to the HT-29 cell line and cholesterol lowering), antimicrobial activities, bile salt hydrolysis, EPS producing capability, heat resistance and resistance to antibiotics.

1.4.2 Potential Health Benefits of Date Fruit

Currently, date fruits have garnered attention due to their associated health benefits, such as antioxidants, antidiabetic properties, cytotoxicity activities, and ACE inhibition. The antioxidant activities of date fruits can be attributed to the presence of minerals like selenium, as well as bioactive compounds such as anthocyanins, carotenoids, phenolic acids, and flavonoids (Al-Mssallem et al., 2020; Echegaray et al., 2021). Moreover, several studies have reported the hypoglycemic effects of date fruits (Chakroun et al., 2016; Djaoud et al., 2022; Singh et al., 2012). This capacity may be ascribed to the modulation of molecular and metabolic pathways by phytochemicals found in date fruits, including steroids, saponins, flavonoids, and phenols (Rahmani et al., 2014). Where, these phytochemicals have the ability to regulate pancreatic cell functions by reducing glucose absorption and stimulating insulin secretion (Vayalil, 2012).

Regarding the antihypertensive effect, Vayalil (2012) and Echegaray et al. (2020) have reported that the polysaccharide content in date fruits is responsible for their ability to inhibit the ACE, thereby reducing blood pressure. Date fruits and their by-products have also exhibited anticytotoxicity activity (Habib et al., 2022; Zhang et al., 2017). From this aspect, quercetin, proanthocyanidins, and luteolin present in date fruits play a role in the antiproliferation effect against certain human cancer cells (Fernández-López et al., 2022).

Organic acids, Sugars, and volatile compounds, are essential agents that give the fruit its balanced taste by adjusting the sweetness and sourness (Velioglu, 2009). The pH

of the organic acid content in fruits acts to regulate the acidity, in addition to other agents (e.g., the amount of undissociated acid), that contribute to each other to achieve the final organoleptic properties of the fruit (Batista-Silva et al., 2018; Vallarino & Osorio, 2019). Furthermore, it is well-documented that organic acids have effects as antimicrobial agents, flavorants, acidity regulators, preservatives, antioxidants, and facilitators of digestion (Adamczak et al., 2020; Martins et al., 2021; Sauer et al., 2008). During fermentation, yeast cells transform sugars derived from fruits into ethanol and carbon dioxide. Simultaneously, numerous secondary compounds are generated, which have an impact on the aroma and taste of the food (Dzialo et al., 2017). This suggests that yeast could induce changes in the volatile profiles of date-pomace. According to the previous studies, the most prevalent categories of volatiles in date fruits are alcohols, esters, aldehydes, and ketones, while many of the most prevalent volatiles originate from phenylpropanoid and fatty-acid metabolic pathways (Ali et al., 2018; Amira et al., 2011; El Arem et al., 2012; Khalil et al., 2017).

Chapters 4 and 5 of this dissertation have discussed the assessment of the chemical characteristics (profiles of organic acids, volatiles, and phenols) and the evaluation of the health-promoting properties after *in vitro* digestion of unfermented DPP and fermented DPP. In Chapter 4, the fermenteation was performed using *Pichia cecembensis* and *Issatchenkia orientalis*, while in Chapter 5, *Saccharomyces cerevisiae* and *Pichia kudriavzevii* were used. Furthermore, these chapters have identified the untargeted and carbohydrate metabolomics profiles of the bioaccessible portions of unfermented and fermented DPR.

Chapter 2: Assessment of Yeasts as Potential Probiotics: a Review of Gastrointestinal Tract Conditions and Investigation Methods

Redrafted from

Alkalbani, N. S., Osaili, T. M., Al-Nabulsi, A. A., Olaimat, A. N., Liu, S. Q., Shah, N. P., Apostolopoulos, V., & Ayyash, M. M. (2022). Assessment of Yeasts as Potential Probiotics: A Review of Gastrointestinal Tract Conditions and Investigation Methods. Journal of Fungi, 8(4). doi:10.3390/jof8040365

2.1 Abstract

Probiotics are microorganisms (including bacteria, yeasts and moulds) that confer various health benefits to the host, when consumed in sufficient amounts. Food products containing probiotics, called functional foods, have several health-promoting and therapeutic benefits. The significant role of yeasts in producing functional foods with promoted health benefits is well documented. Hence, there is considerable interest in isolating new yeasts as potential probiotics. Survival in the gastrointestinal tract (Akyüz et al.), salt tolerance and adherence to epithelial cells are preconditions to classify such microorganisms as probiotics. Clear understanding of how yeasts can overcome GIT and salt stresses and the conditions that support yeasts to grow under such conditions is paramount for identifying, characterising and selecting probiotic yeast strains. This study elaborated the adaptations and mechanisms underlying the survival of probiotic yeasts under GIT and salt stresses. This study also discussed the capability of yeasts to adhere to epithelial cells (hydrophobicity and autoaggregation) and shed light on *in vitro* methods used to assess the probiotic characteristics of newly isolated yeasts.

Keywords: Autoaggregation, coaggregation, gastric, intestine, probiotics.

2.2 Introduction

The term ‘probiotics’ refers to ‘live microorganisms that, when administered in adequate amounts, confer a health benefit on the host’ (Hill et al., 2014). Food products containing probiotics are called functional foods and they provide health benefits, such as antihypertensive, hypoglycaemic, antioxidant (Ashaolu, 2020; Reque & Brandelli, 2021; Wang et al., 2021b) and immunomodulatory effects (Dargahi et al., 2020a; Dargahi et al., 2018; Dargahi et al., 2019; Dargahi et al., 2021; Dargahi et al., 2020b). Numerous clinical studies have proven the beneficial effects of probiotics. These benefits could be conveyed either via the consumption of probiotics as a dietary supplement, or via food products, as a probiotic vehicle (Lee et al., 2014; Wong et al., 2015). Increasing awareness of the health attributes of functional foods amongst consumers has corresponded with an increased demand for the characterisation of new isolates that could be identified as novel probiotic microorganisms. As a result, there have been many medical and industrial food interests in isolating new probiotic species/strains with health-promoting benefits (Daba et al., 2021).

The International Scientific Association for Probiotics and Prebiotics published a consensus paper regarding the characteristics of probiotics (Hill et al., 2014). The potential probiotic microorganism must meet several criteria to be considered probiotic. The essential criteria are tolerance to the GIT conditions (Vera-Pingitore et al., 2016) (e.g. acidic/alkaline pH, digestive enzymes and bile acids/salts), attachment to mucus and epithelial cells and sensitivity to antibiotics (FAO/WHO, 2002). More importantly, the probiotic should be tested in clinical trials to be officially named a probiotic. Hill et al. (2014) stated that probiotic properties are related to strain specificity, not to a particular species or genus of microorganisms.

Yeast cells are eukaryotic microorganisms ~10 times larger in size than bacteria, enabling them to act as a steric hindrance against pathogenic bacteria, thus enhancing its prospects to be a probiotic candidate (Czerucka et al., 2007). Several studies have reported the ability of new yeast isolates to resist GIT conditions, tolerate salt stress, adhere to epithelial cells and possess antimicrobial activity against various pathogens (Perricone et al., 2014; Silva et al., 2011; Zahoor et al., 2021). This characterisation led to research progress to employ potential probiotic yeast strains in functional foods

(Amorim et al., 2018; Di Cagno et al., 2020; Greppi et al., 2017; Rai & Appaiah, 2014). However, amongst all yeast genera, only a few *Saccharomyces cerevisiae* strains have been recognised and are available commercially as probiotics for human consumption (Arévalo-Villena et al., 2018). The *in vitro* characterisation of potential probiotic yeasts is an essential preliminary step before clinical trials. The fulfilled probiotic criteria of yeasts pave the way for conducting investigation and validation for animal models and human trials and for using yeast probiotics commercially in functional foods and for therapeutic purposes (Gut et al., 2019).

The use of probiotics in foods or dietary components provides superior health benefits to conventional food products (Ayyash et al., 2019). Ogunremi et al. (2015) reported using the probiotic strain of *Pichia kudriavzevii* OG23 to produce fermented cereal-based food with higher antioxidant activity and various flavour compounds (Ogunremi et al., 2015). The combination of *S. cerevisiae* var. *boulardii* and inulin developed symbiotic yogurt. Sarwar et al. (2019) reported improved product texture and increased amount of desirable volatile compounds. In addition, the probiotic strain *S. cerevisiae* var. *boulardii* is used to produce alcohol-free beer (Senkarcinova et al., 2019).

Probiotic yeasts have also gained importance in promoting animal nutrition and health. In the past, yeast as probiotics was employed as a feed additive because of its rich source of fibre, protein, minerals, organic acids and B vitamins (Golubev, 2006). Adding viable and nonviable yeast cells to animal feed promotes health and growth (Roto et al., 2015). Probiotic yeast (*S. cerevisiae*) has positively impacted poultry health by increasing egg production, improving feed intake and reducing plasma cholesterol (Hassanein & Soliman, 2010; Vohra et al., 2016). In addition, *S. cerevisiae* has been used in ruminant feed to reduce lactate accumulation (Marden et al., 2008). In aquaculture, stimulating the enzymatic antioxidative response of farmed fish has been reported using *Debaryomyces hansenii* as a dietary supplementation (Reyes-Becerril et al., 2008).

Despite the importance of yeasts in food industries, they could contribute to the spoilage of different foods and might be pathogenic for the host. The traditional identification methods of microorganisms based on physiological, biochemical and

morphological characteristics are insufficient and inaccurate to classify the yeast as nonpathogenic. Conventional methods are time-consuming and require significant human skills. Therefore, the characterisation of yeasts at the genomic level might be decisive in distinguishing between nonpathogenic and pathogenic yeast strains. Molecular methods, such as polymerase chain reaction-based techniques, mitochondrial DNA restriction analysis and chromosome electrophoretic analysis, have been effectively used in yeast strain identification (Andrighetto et al., 2000; Querol & Ramon, 1996). Furthermore, rapid and reliable fragment analysis tools have been established to identify foodborne yeasts, such as random amplified polymorphic DNA, pulsed-field gel electrophoresis and restriction fragment length polymorphism (Arastehfar et al., 2019; Chen et al., 2009b; Liu et al., 2018b; Prillinger et al., 1999; Soll, 2000).

The applications of yeast probiotics have been addressed in several reviews (Kunyeit et al., 2020; Saber et al., 2017; Shurson, 2018). However, the assessment methods of potential probiotic characteristics of newly isolated yeasts have not been addressed. Therefore, there is a need for a comprehensive review of the assessment methods of new isolated yeasts as potential probiotics and the appropriateness of these *in vitro* assessment methods used. Thus, this review identified the newly isolated yeasts characterised as potential probiotics, assessed the characterisation methods and highlighted the mechanistic effects of the assessment methods on yeasts.

2.3 The Gastric Environment

Digestion is the process that degrades food substances into nutrients (e.g. phytochemicals, micronutrients and macronutrients) to release them into the bloodstream (Liu et al., 2020a). This process comprises mechanical and chemical digestion. Mechanical digestion breaks down food substances into small particles as a prelude to effective chemical digestion (Camilleri, 2006), whereas chemical digestion is responsible for catabolising other food molecules via various digestive enzymes to absorb them into the bloodstream (Welcome, 2018). Peristaltic contractions of the smooth muscle display mechanical digestion in the stomach, consisting of propulsion, churning and grinding (Welcome, 2018). Only tiny particles (<2 mm) can enter the duodenum (Camilleri, 2006). The bigger particles are churned back towards the stomach for further mechanical

and chemical digestion (Meyer et al., 1981). The stomach provides an environment for a fundamental part of chemical digestion. The gastric mucosa contains two glands, the so-called oxyntic and pyloric glands, associated with chemical digestion (Carlson, 2019).

Oxyntic glands are found in the stomach body. Their parietal cells produce hydrochloric acid (HCl; 160 mmol/L, pH 0.8) (Herdt, 2020). HCl is important to exterminate pathogenic microorganisms ingested together with foods or drinks (Schubert, 2003) and to activate pepsin from the zymogen pepsinogen, which is secreted by the chief cells of oxyntic glands (Carlson, 2019). Another intrinsic function of HCl is protein denaturation to facilitate enzymatic digestion by pepsin. Pepsin breaks down the peptide bonds of proteins at the optimal pH of 2.0–3.0 into individual amino acids, which are released into the bloodstream (Camilleri, 2006; Carlson, 2019). Pyloric glands are located in the stomach antrum (Schubert, 2017). They form the hormone gastrin secreted by their enteroendocrine G-cells, which act to induce the creation of HCl (Herdt, 2020; Schubert, 2017). In addition, mucus secreted by mucous cells of pyloric glands plays a significant role in protecting the gastric surface from the acidic medium in the stomach (Berin et al., 2014).

2.3.1 Tolerance to Gastric Conditions

Several studies have defined the growth inhibition of microorganisms subjected to GIT conditions (da Silva Guedes et al., 2019; Rui et al., 2019; Sekova et al., 2018). Acids can diffuse passively through the cell membrane and thus access the cytoplasm, dissociating into charged derivatives and protons (Guerzoni et al., 2013). Proton accumulation in the intracellular cytoplasm may decrease the intracellular pH and subsequently affect the transmembrane pH gradient (Orij et al., 2011), which contributes to the proton-motive force and minimises the amount of energy obtainable for cellular growth (Guan et al., 2020; Horák, 1997). Furthermore, internal acidification reduces the inhibition of the action of acid-sensitive microorganisms, but extremely low internal pH induces damage to DNA and enzymes and denatures proteins, resulting in cell death (Estruch, 2000; Jaichumjai et al., 2010). Besides accumulating dissociated organic acids in the cytoplasm, this condition has a destructive effect on cellular physiology (Viljoen, 2006). Gastric stress experiments with different potential probiotic yeasts are

summarised in Appendix A.1 (pH 2.0, 0.0133 g/L pepsin, 2.5 h and 37°C). The survival rate of *Metschnikowia pulcherrima* isolated from fermented table olives reached 96.4% (Bonatsou et al., 2018). This survival percentage was relatively lower in *Candida adriatica* collected from olive oil (23.4%) (Zullo & Ciafardini, 2019).

Figure 1 illustrates the percentage of the total number of isolated yeast genera subjected to gastric and intestinal conditions. The boxplot chart of Figure 2 displays the percent survival rate under gastric conditions. Although *Candida* did not exhibit the highest survival rate (Figure 2), it was the most frequently isolated genus at 34%, followed by *Pichia* and *Saccharomyces* (14% for each) and *Aureobasidium* (7.6%; Figure 1).

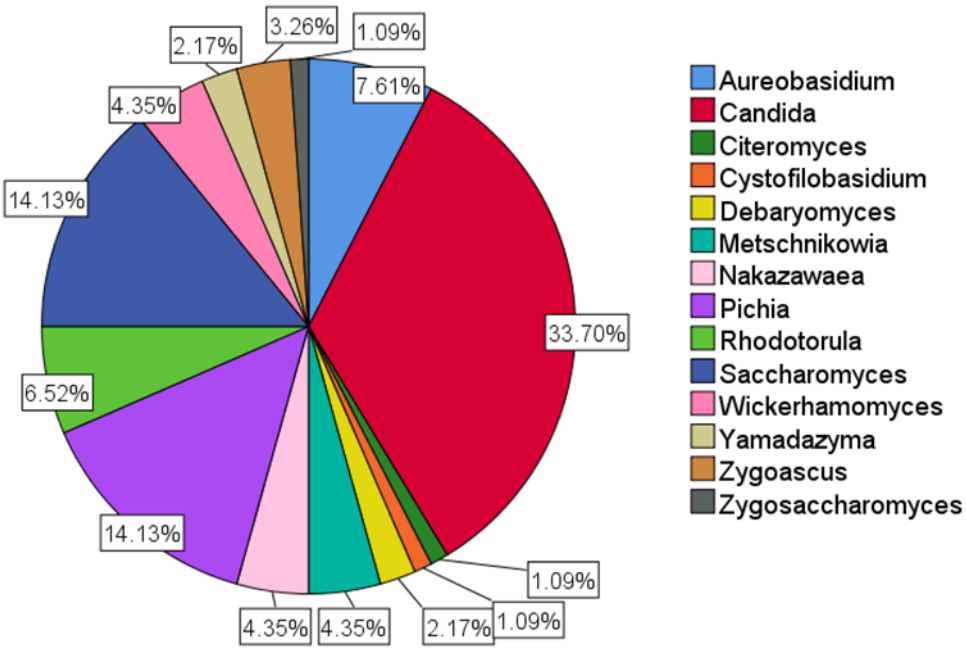

Figure 1: Total number (%) of isolated yeast genera under gastrointestinal conditions.

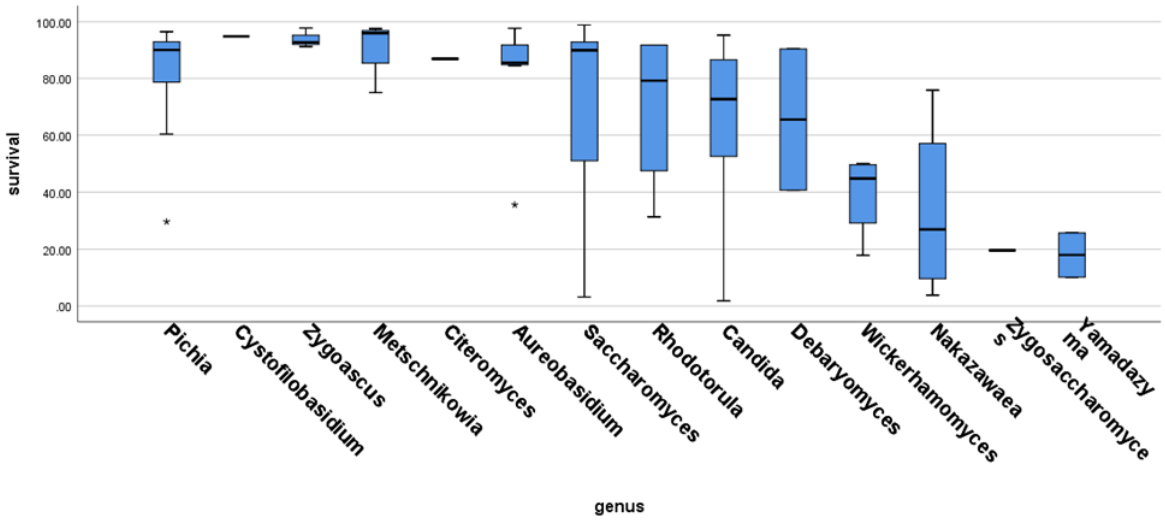

Figure 2: Survival rate (%) of yeasts under gastric conditions in descending order according to genera.
* is outliners.

2.3.2 Assessment Methods of Gastric Tolerance

Three factors must be considered when adopting such a method for screening GIT stress tolerance. These factors are derived from the digestion process *in vivo*: (1) diffusion of gastrointestinal fluids into the food matrix, (2) synchronisation of mechanical digestion with chemical digestion and (3) sequence of enzymatic degradation. Therefore, time is a decisive parameter.

The significant challenges in the stomach are the extreme acidity, pH from 2.0 to 3.0 and the presence of digestive enzymes, such as pepsin (Palla et al., 2019). This condition inhibits most microorganisms, including yeasts (Puppala et al., 2019). To qualify as probiotics, yeast strains have to survive the gastric conditions and to reach the gut alive, where they will exert their function (Ragavan & Das, 2020). Some microorganisms, including yeasts, possess the capability to survive and grow in an acidic medium. The survival of selected yeast strains against digestion conditions is usually evaluated *in vitro* in a gastric-like environment, where simulated gastric juice is prepared in a buffer solution at a low pH level (preferably pH 2.0) in the presence of pepsin for given time intervals at 37°C (Porru et al., 2018). This method aims to quantify strain viability after being subjected to gastric juice. However, many researchers (Du Toit et al., 1998; Kenfack et al., 2018; Zahoor et al., 2021) used only acidic pH without adding pepsin to evaluate pH tolerance. They overlooked assessing gastric tolerance as a

precondition to consider such microorganisms as probiotics, leading to inaccurate outcomes.

For all studies in this review (Appendix A.1), a buffer solution consisting of NaCl, KH_2PO_4, $CaCl_2$ and KCl was used. Cells of each yeast strain were resuspended in gastric fluid and incubated for 2.5 h at 37°C (Bonatsou et al., 2015; Bonatsou et al., 2018; Oliveira et al., 2017; Porru et al., 2018; Zullo & Ciafardini, 2019). Given the importance of mechanical movement, no studies (Appendix.A1) mentioned the application of mechanical movement while assessing the gastric tolerance of potential yeast probiotics as a part of their assays, which could result in an imprecise assessment.

2.3.3 Mechanisms of Gastric Tolerance

The underlying mechanism for surviving yeast under low pH is modifying the yeast cell wall (Kapteyn et al., 2001; Lucena et al., 2020). Strong inorganic acids, such as HCl, in the stomach and yeast cells adjusted to low pH comprise a mechanism that activates the cell wall integrity pathway (Chen et al., 2009a; de Lucena et al., 2015). It depends on the transmission of the signals of stressed walls to Rho1 GTPase, leading to the formation of various carbohydrate polymers used for remodelling the cell wall (Levin, 2011). Another mechanism for resisting the strong inorganic acid is a general stress response pathway, an essential response against any conditional alteration, where the conductance of the protein kinase C pathway is fundamental (Lucena et al., 2020).

In addition, under low pH, the temporal inefficiency of the glucose sensing pathway in yeast and growth inhibition are the main causatives of the activity reduction of protein kinase A, thereby liberating general stress responses and driving to remodel cell gene expression to adjust to the low-pH condition (de Lucena et al., 2015; De Melo et al., 2010; Lucena et al., 2020). Furthermore, calcium metabolism could affect yeast responses to low external pH (Brandão et al., 2014), in which the deletion of either Mid1p or Cch1pm as calcium channels is vital to yeast cells when subjected to inorganic acid stress (Claret et al., 2005; De Lucena et al., 2012).

Adjusting the membrane lipid composition in *Candida glabrata* by mediator subunits led to increases in acid tolerance up to pH 2.0, indicating the significant role of

lipid homoeostasis (Lin et al., 2017). Fletcher et al. (2017) proved that acid tolerance could be acquired by altering sterol composition and reducing iron uptake, such as in *S. cerevisiae*, as its acidic tolerance developed to pH 2.8. In line with previous studies, the use of pepsin and low pH (2.0) is recommended to mimic human gastric conditions and thus obtain an accurate assessment when examining new isolates in gastric juice.

2.4 Intestinal Conditions

The small intestine is associated with pancreatic enzymes and bile released from the liver to continue digestion. The secretions of the pancreas and duodenum mix with digesta and chyme (Camilleri, 2006). As peristalsis occurs, mechanical digestion goes on slightly. The small intestine environment is neutral to slight alkalinity because of the bicarbonate produced by the pancreas. It allows the digestive enzymes secreted by the duodenum and pancreas (e.g., pancreatic amylase, pancreatic lipase and trypsinogen) to act optimally at pH 6–7 (Kiela & Ghishan, 2016). Pancreatic amylase is responsible for hydrolysing starch into maltose and maltotriose (Silk, 1986). At the same time, pancreatic lipase is associated with a coenzyme named colipase for hydrolysing triglycerides to produce diacylglycerols and monoacylglycerols (Alexander, 2009; Silk, 1986). Trypsinogen is a zymogen of trypsin. It is converted to its active form (trypsin) by enterokinase, and trypsin converts other pancreatic zymogens to their active forms (Chen et al., 2013).

Pancreatic zymogens transfer to the common bile duct creating the ampulla of Vater and empty its contents into the duodenum, where pancreatic zymogen activation occurs. Liver cells form bile, which is stored in the gallbladder and carried by the common bile duct (Semrin & Russo, 2010). Bile contains a mixture of bile salts, fatty acids, cholesterol, electrolytes and bilirubin that make the total solution basic with an average pH of 8.2 (Drury et al., 1924). Bile salts and acids pass to the small intestine, where they function as detergents for waste products from the blood and are critical for breaking down fat into fatty acids (Maldonado-Valderrama et al., 2011).

2.4.1 Tolerance to Intestinal Conditions

The major obstacles to yeast survival are high bile salt and organic acid concentrations. Moreover, Pais et al. (2020) highlighted that pancreatic and hydrolytic enzymes, secondary metabolic products of the gut microbiome and epithelial brush border in the small intestine, might destroy microorganisms, including yeasts. Bile salts are generally created from cholesterol in the liver and secreted into the intestine to contribute to the digestive process (Aguilar-Ballester et al., 2020). Previously, Urdaneta & Casadesús (2017) stated that bile salts have detergent properties, and as a result, they can be toxic to the GIT microbiota. However, some microorganisms resist bile salts and hydrolytic enzymes (Merchán et al., 2020).

Alkaline stress is another challenge that inevitably exposes yeast strains in the intestinal tract. For example, *S. cerevisiae* grows in acidic pH better than in alkaline conditions (Chen et al., 2009a). These strains do not proliferate when pH exceeds 8.0–8.2 (Canadell et al., 2015). Indeed, *Schizosaccharomyces pombe* growth was inhibited even at neutral pH (Tominaga et al., 2019). Nevertheless, some yeast species, such as *Yarrowia lipolytica*, are resistant to alkaline conditions up to pH 10–11 (Peñalva & Arst, 2004; Zvyagilskaya et al., 2001). Previous studies confirmed that pH changes lead to upregulated and downregulated gene expression in *S. cerevisiae* (Causton et al., 2001; Serrano et al., 2006; Serrano et al., 2002).

The active transporters of the plasma membrane are activated by the permanent proton gradient between extracellular and intracellular media, which is protected by respective fungal orthologs, for instance, in *S. cerevisiae* by Pma1p H^+-ATPase (Skoneczny & Skoneczna, 2018). The importance of this gradient comes from its functional uptake of several vital compounds (Guan et al., 2020). Acidic stress affects it slightly, but alkaline stress damages it (Skoneczny & Skoneczna, 2018). When cells cannot neutralise a circumambient alkaline medium, microorganisms suffer from starvation for nutrients, such as glucose and phosphate (Casamayor et al., 2012; Serra-Cardona et al., 2014). In addition, extreme external pH reduces the ionisation of primary transition metals, leading to starvation for these metals (e.g. iron and copper) (Higuchi et al., 2018).

Appendix A.2 summarises various yeast strains undergoing *in vitro* intestinal conditions (pH 8, 0.1 g/L pancreatin, 3.0 g/L bile salts, 3.5 h and 37°C). Bonatsou et al. (2015) tested the response of *Rhodotorula diobovatum* isolated from Greek black olives, which displayed only a 3.83% survival rate. Other species isolated from the same source (*Rhodotorula mucilaginosa*) reached 53.40%. The differences in the survival rate were species- and strain-dependent.

The percentage of the total number of isolated yeast genera undergoing intestinal conditions is presented in Figure 1 (pH 8, 0.1 g/L pancreatin, 3.0 g/L bile salts, 3.5 h and 37°C). Figure 3 shows that *Cystofilobasidium* achieved the highest survival rate, whereas *Zygoascus* occupied the second rank, followed by *Metschnikowia* and *Pichai*, which relatively retracted compared to their position in gastric tolerance.

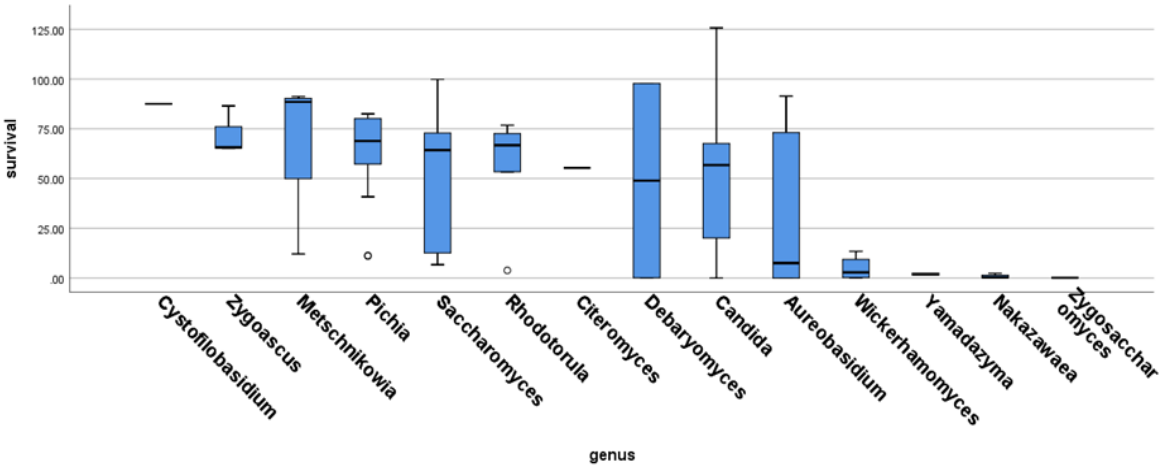

Figure 3: Survival rate (%) of yeasts under intestinal conditions.
o = outliners.

2.4.2 Assessment Methods of Intestinal Tolerance

Intestinal tolerance is a prerequisite to consider yeast as a probiotic, in addition to its survival under notable pH and temperature variations, through their passage from the stomach to the small intestine (Sen et al., 2020). The assessment of intestinal tolerance of the potential yeast probiotic is mainly performed based on a similar concept to the *in vitro* gastric condition test. The optimum concentration of the bile in the human gut environment and the actual human temperature range are 0.3–0.6% and 36.5–37.5°C, respectively (Helmy et al., 2019). Thus, isolated yeasts are commonly incubated at 37°C for ~3.5 h after resuspension in an intestinal-like fluid containing pancreatin and bile salts at pH 8.0. Both sodium phosphate dibasic heptahydrate and sodium chloride were used to make a buffer solution for experiments of intestinal tolerance in recently reviewed studies.

The INFOGEST *in vitro* digestion model (Figure 4) is widely applied in food research. This method includes three successive stages that simulate the digestion process in the upper GIT *in vivo*. The modality to implement INFOGEST is based on pH values, enzyme activity and the ionic strength of electrolyte solutions used in oral, gastric and intestinal phases. Furthermore, the digestion durations are 2.0, 120 and 120 min, respectively, under agitation status (Brodkorb et al., 2019). Thus far, according to the three factors mentioned in Section 2.2, the INFOGEST static model is considered a more reliable method to evaluate the GIT stress tolerance for microorganisms because it is almost the most simulated *in vivo* GIT. However, to the best of the authors' knowledge, up to now, there is no application of the INFOGEST assay on the assessment of the GIT stress tolerance of yeast probiotics. By contrast, screening for this essential probiotic criterion of a potential probiotic bacterium by the INFOGEST model has been applied by many researchers (Ayyash et al., 2021a; Gomez-Mascaraque et al., 2016; Ta et al., 2021).

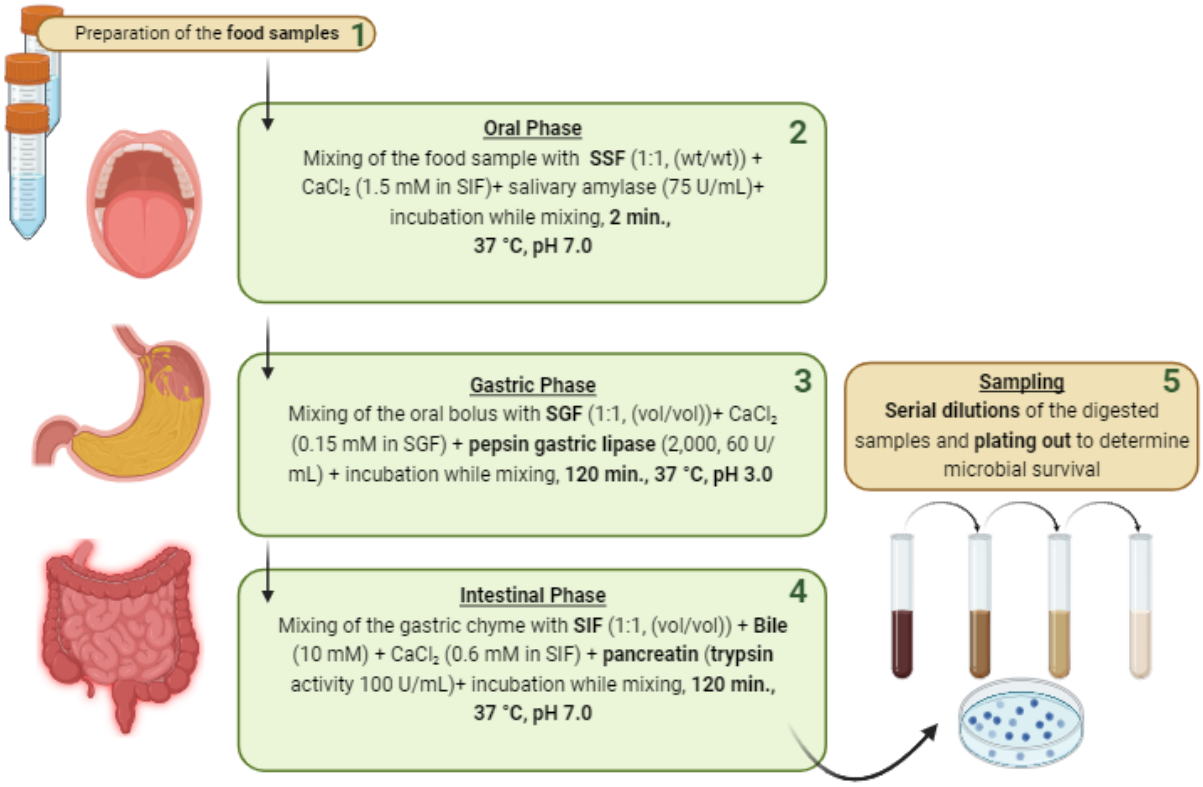

Figure 4: Schematic in vitro digestion method. SSF, simulated salivary fluid; SGF, simulated gastric fluid; SIF, simulated intestinal fluid (Created with BioRender.com).

2.4.3 Mechanisms of Intestinal Tolerance

According to FAO/WHO (2002), resistance to bile acids/salts is a precondition for probiotic yeast to survive in the small intestine through the passage in the GIT. Bile salts have strong toxic effects on the cellular membrane of microorganisms (Begley et al., 2006) in terms of their fluidity, charge and hydrophobicity, perturbing cellular homoeostasis and motive oxidative stress (Begley et al., 2005). However, several microorganisms can overcome the toxic effects of bile salts by employing bile salt hydrolases (BSH) of the intestinal microbiome, minimising the toxic influence of conjugated bile (Elkins et al., 2001). The mechanism of bile resistance in yeast is not entirely understood (Martins et al., 2008). Despite that, bile salt resistance in probiotic yeasts has been shown by several studies (Hossain et al., 2020; Zahoor et al., 2021). Recently, a BSH, an enzyme responsible for hydrolysing bile salts, was investigated *in vitro* and detected in *Saccharomyces boulardii* (Hernández-Gómez et al., 2021), which might contribute to understanding yeast bile resistance.

Numerous mechanisms have been suggested to explain yeast resistance regarding yeast alkaline tolerance. One of them occurs in *S. cerevisiae*, where yeast plasma membrane Ena1 P-type ATPase acts on the cell's efflux K^+ and Na^+ cations, counteracting the internal alkaline medium (Nakayama et al., 2004; Skoneczny & Skoneczna, 2018). Another mechanism in *S. cerevisiae*, the protein kinase A pathway, is inhibited under alkaline stress, leading to the remodelling of *Msn2* and *Msn4* gene expression of stress-responsive transcriptional activators, to offset environmental alkalisation (Casado et al., 2011). Moreover, Casamayor et al. (2012) documented that *S. cerevisiae* resorts to glycogen mobilisation to respond to alkaline pH stress to compromise glucose uptake. The vacuolar H^+-ATPase enzyme is also essential to regulate intracellular pH in yeast cells (Diab & Kane, 2013; Wilms et al., 2017). Another strategy is adopted by *Y. lipolytica* to cope with alkaline stress using polyphosphate storage molecules. The elevated pH of the cytosol drives polyphosphate hydrolysis, which compensates for the phosphate storage and restores the proper pH of the intracellular medium (Yang et al., 2017; Zvyagilskaya et al., 2001). Simulating the intestinal fluid in the presence of pancreatin bile salts, preparing them at a similar concertation to the natural digestive system and setting a pH range from 7.8 to 8.0 are keys to an accurate appraisal of new isolates against intestinal juice.

2.5 Salt Conditions

In several food processes, probiotic cells are constantly subjected to environmental stresses, including excessive salinity, which forms hyperosmotic stress (salt stress). Probiotic yeasts could be exposed to hyperosmotic stress during the food production process, such as in the production of fermented food and certain cheese varieties (Capusoni et al., 2019; Helmy et al., 2019).

In recent years, there have been a variety of food matrices targeted as probiotic vehicles containing significant amounts of NaCl (Deparis et al., 2017). For instance, a high-brine solution has been used as a flavour improver and preservative agent (Gandhi & Shah, 2016). Fermentation of black olives occurs in 80–100 g/L brine (Romero-Gil et al., 2013), whereas the salt concentration in soy sauce reaches up to 18% (Hou et al., 2013) or higher. During the fermentation process, salt can repress the growth of moulds

and some yeasts, the main causative agents of food spoilage, and can suppress the growth of specific foodborne pathogens, such as *Listeria monocytogenes* and *Staphylococcus aureus* (Song et al., 2015; Yao et al., 2020). Further, salt preservative properties are associated with shelf-life extension, flavour improvement and fermented food products (Fleet, 2011b; Gandhi & Shah, 2016).

2.5.1 Salt Stress

Salt tolerance can be a critical feature for selected yeast strains, thus partially qualifying them as probiotics in traditional food fermentation or processing. However, an elevated saline environment can damage enzyme structure, suppress metabolic enzyme activity and retard fermentation (Hong et al., 2013). Moreover, Heinisch & Rodicio (2017) documented that high salinity may drive cell plasmolysis, and intracellular water molecules could diffuse out of the cells. Hence, owing to the lack of timely and efficient responses, high-salt conditions could cause growth inhibition or yeast death (Hohmann, 2015). However, some yeast strains can survive and grow under such an environment, relying on strain-specific abilities to detect and respond to salt stress (Sharma & Sharma, 2017). Appendix A.3 presents the assessed non-inhibitory concentration (NIC) and minimum inhibitory concentration (MIC) values (g/L) measured under NaCl conditions for some yeast strains. *S. cerevisiae* isolated from Kalamata table olive fermentation and subjected to NaCl tolerance test at pH 3.5, 5.0 and 6.5 showed MIC values of 146.73, 174.8 and 143.8 g/L, whereas the same source was used to collect *Zygoascus hellenicus* and demonstrated MIC values of 125, 147 and 129 g/L.

From the same data, *Candida* was the most isolated genus (35%) amongst the 13 genera (Figure 5). Although it did not achieve the highest mean MIC value (Figure 6), which was recorded for *Wickerhamomyces*, it was not halophilic.

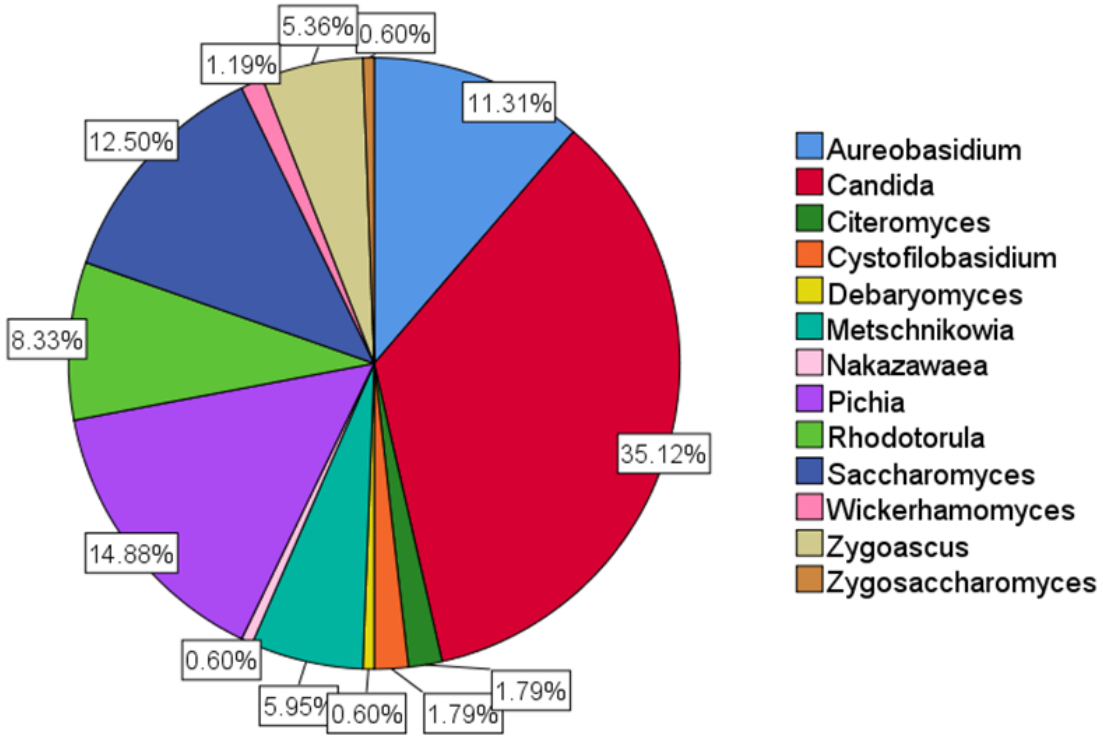

Figure 5: Total number (%) of isolated yeast genera after NaCl stress.

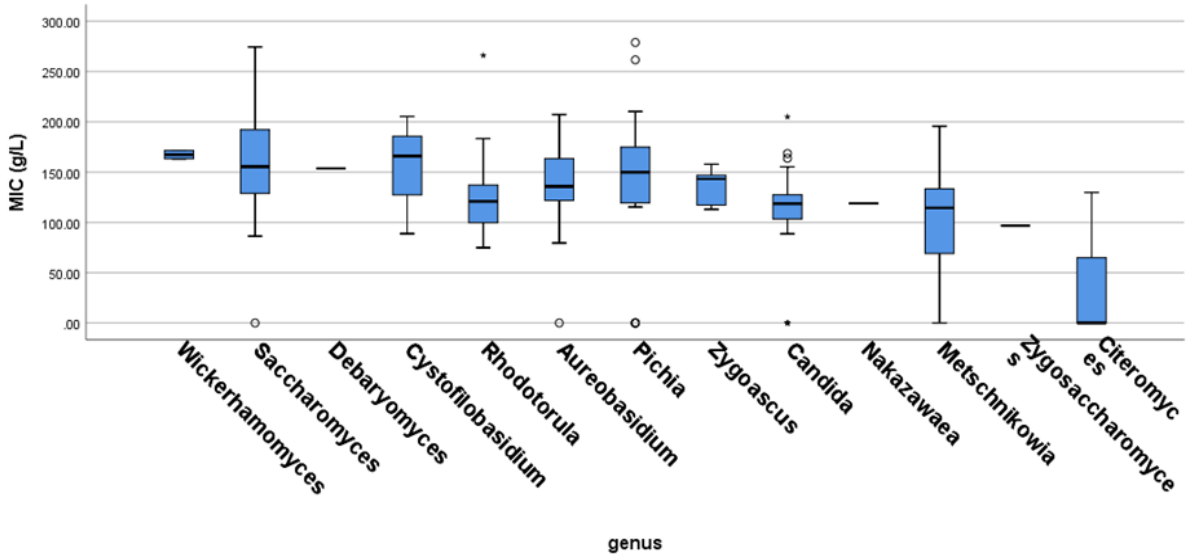

Figure 6: MIC (g/L) of yeasts under NaCl conditions in descending order according to genera.
o = outliners.

2.5.2 Assessment Methods of Salt Tolerance

Lately, salinity tolerance test was carried out *in vitro* by adding yeast suspensions to appropriate broth (e.g., yeast malt broth) supplemented with different NaCl concentrations (e.g., 0–250 g/L) at various pH values (e.g., 3.5, 5.5 and 6.5). The growth of the strains was measured as optical density (OD) at 420 nm in an automatic spectrophotometer for a specific period (e.g., 12 h for a fixed interval, such as 7 days). The NIC and MIC at different NaCl/pH values for each period were calculated by comparing the area of the curves under OD/time for the control where salt was absent to the area of the curves where salt at different pH values was used (Bonatsou et al., 2018; Porru et al., 2018). Because salt is used in many processed foods, the inaccuracy in assessing its tolerance may suppress the growth of putative probiotic strains in the food product. Accordingly, yeast strains subjected to salt stress for short periods (2 h) may not give correct information regarding the ability of the strains to tolerate salt Bonatsou et al. (2015).

2.5.3 Mechanisms of Salt Tolerance

Hyperosmotic stress induces water efflux, resulting in cell shrinkage, excessive cellular solute concentrations and abolished cell turgor pressure (Hohmann, 2015; Pascual-Ahuir et al., 2018). Microorganisms counteract such influences by coping with various energetic mechanisms that modify cellular energy homoeostasis and reduce microbial growth (Yao et al., 2020). Cells adjust their intracellular osmotic pressure by producing osmolytes, which are small organic solutes (such as glycerol and trehalose) that help in retaining intracellular water (Dakal et al., 2014; Hounsa et al., 1998).

Hyperosmotic stress tolerance in yeasts is adopted via the high osmolarity glycerol (HOG) pathway, where mitogen-induced protein kinase is activated by leveraging two cell membrane-bound receptors (Brewster & Gustin, 2014; Pascual-Ahuir et al., 2018). More specifically, sho1p and sln1p reveal osmotic alterations, leading to the stimulation of HOG pathway genes (Rodrigues-Pousada et al., 2004; Skoneczny & Skoneczna, 2018) and resulting in downstream triggered genes related to salt stress resistance (Dhar et al., 2011). When conducting a salt tolerance test to evaluate novel probiotic yeasts, it is recommended to apply different NaCl concentrations at

various pH values to estimate each environment in fermented food matrices and targeted food processing protocols. The results could indicate the strains that may survive and dominate during fermentation.

2.6 Autoaggregation

Autoaggregation is associated with promoting colonisation in the human intestine, immunomodulation of the colonic mucosa and prevention of pathogenic infections (Isenring et al., 2021). Intestinal epithelial cells are covered by the mucosal glycocalyx layer that mainly contains electrolytes, immunoglobulins, glycolipids and glycosylated proteins (mucins) with sugar residues (Monteagudo-Mera et al., 2019). Adhesion property is gained by particular cell-surface proteins named ‘flocculins’ or ‘adhesions’ on the yeast cell surface that bind sugar residues, as on mucins in epithelial cells or certain amino acids on the surface of the other cells (Verstrepen & Klis, 2006).

The autoaggregation property is represented as the ability of yeast strains to self-aggregate and produce flocs as a survival response, which extends a competitive advantage over other microorganisms, including enteric bacterial pathogens in severe conditions, such as human GIT (Gut et al., 2019). This ability is an essential property of selected probiotics. Fernandez-Pacheco Rodríguez et al. (2018) noted that probiotics should adequately adhere to epithelial cells to colonise the intestinal mucosa and exert its functional effects. In addition, the creation of cellular aggregates expands cell protection in an adverse environment (Suvarna et al., 2018). Because yeast cells are larger and heavier than bacteria, they sediment faster and in a higher amount (Lima et al., 2017).

The outlines of the autoaggregation capacity of the selected yeast strains, measured in different periods, are shown in Appendix A4. *Candida molendinolei* isolated from Kalamata table olive fermentation exhibited 42%, 48% and 93% autoaggregation percentages after 2, 4 and 24 h of incubation. After 4–24 h of incubation, the yeast strains continuously increased their capability to make cellular aggregates. *Candida tropicalis* is another *Candida* species collected from fermented Portuguese table olives, demonstrating ~ 86% autoaggregation. *Nakazawaea wickerhamii* isolated from olive oil displayed only 18%. This variance showed that the autoaggregation capability is substantially strain-dependent (García-Cayuela et al., 2014). In another perspective, the

most frequently isolated genus amongst yeast genera was *Candida* with 27.7%, followed by *Saccharomyces* and *Pichia* with 19.3% and 16%, respectively (Figure 7), whereas the highest autoaggregation percentage was exhibited by *Aureobasidium* and *Saccharomyces* (Figure 8).

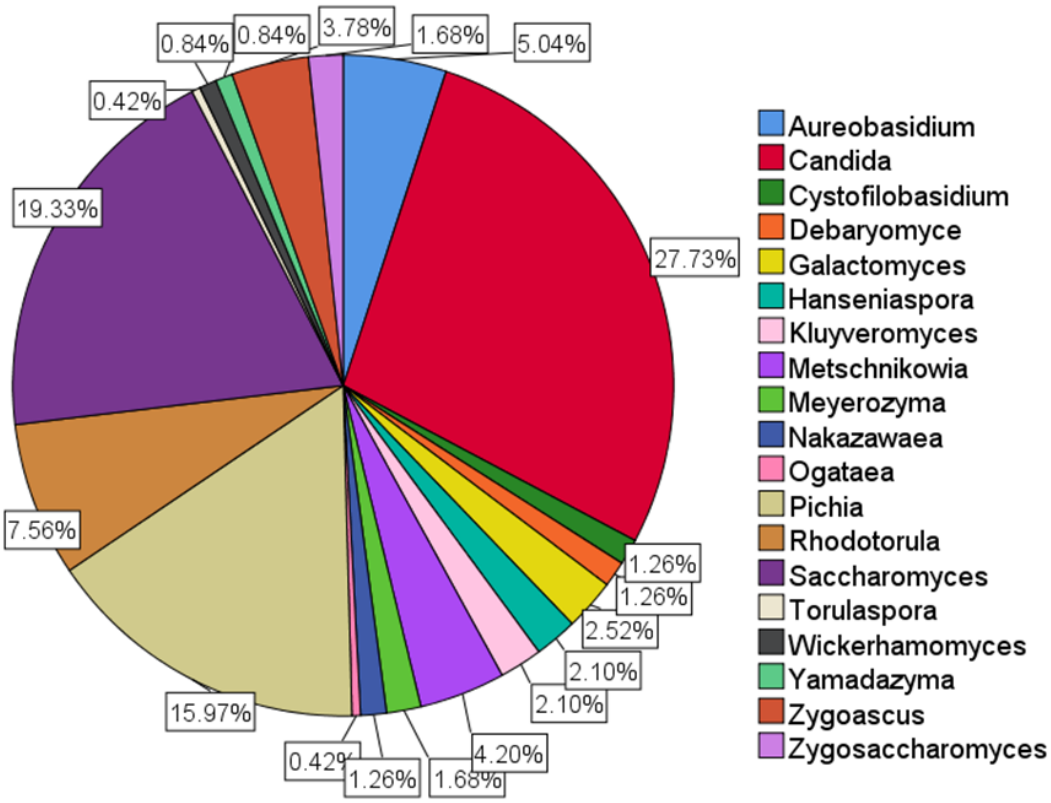

Figure 7: Total number (%) of isolated yeast genera that underwent the autoaggregation test.

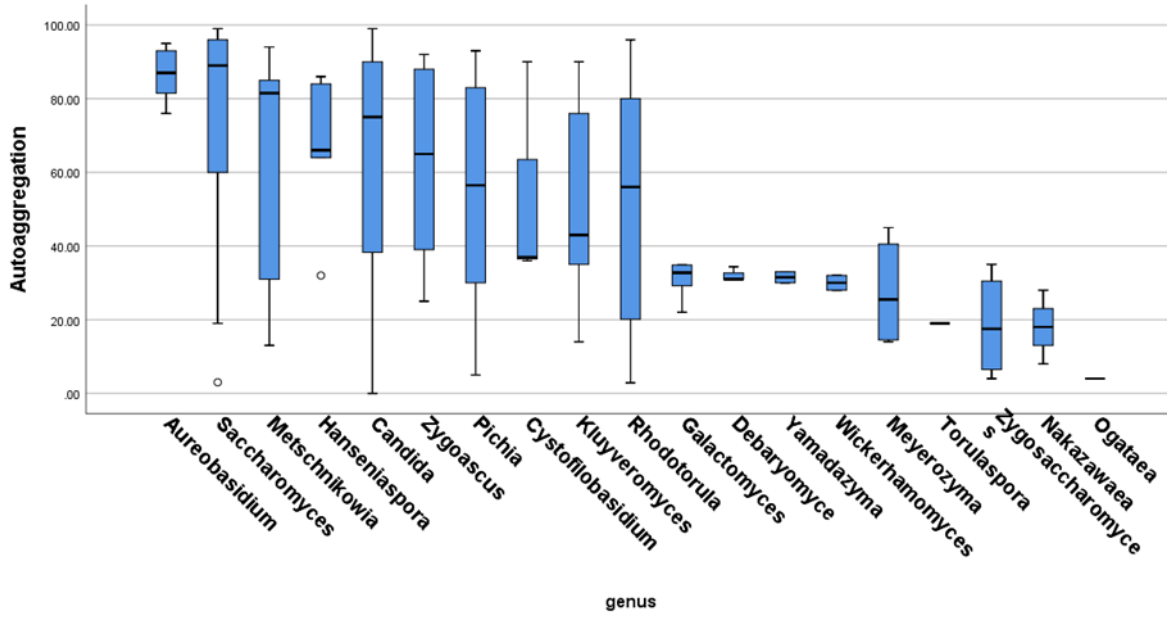

Figure 8: Autoaggregation (%) in descending order according to yeast genera.
o = outliners.

2.6.1 Assessment Methods of Autoaggregation

Generally, the autoaggregation percentage of selected yeast strains proceeds *in vitro* via the resuspension of the suspended strains in phosphate-buffered saline (PBS) or NaCl solution (mostly 0.9%, w/v), and the test is carried out in a disposable plastic cuvette. The absorbance is measured at 600 nm using an automatic spectrophotometer at different time intervals without shaking the cell suspensions (Bonatsou et al., 2018; Oliveira et al., 2017). Yeast strains isolated by Gut et al. (2019) and Fernandez-Pacheco Rodríguez et al. (2018) had undergone autoaggregation evaluation for only 2 h or 30 min, respectively. Quantifying the autoaggregate capacity of yeast strains under a short time could not reflect their actual capacity. *In vitro*, the median transit time through the gut is 28.7 h (Asnicar et al., 2021; Nandhra et al., 2020), and the mean short intestinal transit time is 4.2 h (Roland et al., 2015), where the autoaggregation phenomenon is expected to activate its function.

2.6.2 Mode of Action of Autoaggregation

Autoaggregation (or, to be exact, ‘flocculation’) in yeasts is a complex phenomenon that takes place predominantly upon sugar depletion throughout the plated exponential or stationary phase (Gil-Rodríguez et al., 2015). In this circumstance,

autoaggregation is influenced by the differences in cell wall composition, the presence and morphological type of cell appendages and the protruding macromolecules from the cell wall (Stewart, 2018).

Di Gianvito et al. (2017) demonstrated that flocculation occurs in two main phases: equal cell–cell adhesion is formed on glycan–glycan interaction and the glycan–lectin binding. First, glycan–glycan interaction is fixed by specific proteins known as flocculins, zymolectins, adhesins or lectins (lectin-like theory) (Miki et al., 1982). Then, residual mannose in the cell wall is bound by any proteins that protrude from the cell surface (Touhami et al., 2003). Ca^{2+} ions in the environment are significant because of their contribution to sugar binding and maintaining the correct flocculin conformation (Goossens et al., 2015; Miki et al., 1982). To gain the correct autoaggregation result, it is advisable to perform the test at gradual time intervals and suspend the strains in a saline buffer solution at the simulated NaCl concentration in the human GIT.

2.7 Hydrophobicity

Another property of potential probiotic strains is cell surface hydrophobicity. As in autoaggregation, hydrophobicity is crucial in reflecting the ability of probiotic adhesion and colonisation in the epithelial cells of the human GIT, where they may extend prophylactic and therapeutic impacts (Liu et al., 2020b), resulting in the prevention of pathogen colonisation by their interactions (Somashekaraiah et al., 2019). The tendency of microorganisms to adhesion can increase according to the surface type (Krasowska & Sigler, 2014). Thus, cells with high hydrophobic properties attach more strongly to hydrophobic surfaces (Kochkodan et al., 2008). Surface hydrophobicity in mammals, including humans, is very high on the top of the gastric mucosa and the colon (Lugea et al., 2000). This hydrophobic property is attributed to the surface-active phospholipid layer that lines the mucus top that coats the epithelium (Farhadi et al., 2003). Van Tassell & Miller (2011) stated that, initially, the reversible and weak physical binding between probiotics and mucosa has occurred through nonspecific contact by hydrophobicity and spatial recognition. Subsequently, stable binding has been established between probiotic adhesins, usually surface-anchored proteins and

complementary receptors in the mucus or intestinal epithelial cells, effectively colonising the GIT (Van Tassell & Miller, 2011).

The correlation between the microbial ability to colonise the GIT and hydrophobicity has been studied *in vivo* and has been debated widely (Ehrmann et al., 2002; Hill et al., 2014; McGinnis, 2004). Nevertheless, this is not a compulsory characteristic as yeasts are commonly excreted in the faeces because of competition with the GIT microbiota (França et al., 2015). Therefore, probiotic yeasts should be regularly consumed to conserve appropriate balance in the host track, considering that the probiotic impact is dose-dependent (FAO/WHO, 2002).

The hydrophobicity of the studied yeast strains towards n-hexadecane is presented in Appendix A.5. Interestingly, all strains isolated from fermented foods by Menezes et al. (2020) resulted in > 90% hydrophobicity, confirming that fermented foods could serve as carriers for probiotic microorganisms (Rezac et al., 2018), including yeast. From another viewpoint, *Saccharomyces* is the most often isolated genus (Figure 9), followed by *Pichia* and *Candida*, regarding the ratio of the isolated yeast genus. However, these latter two genera hold the fifth (55.47%) and sixth (45.35%) positions, respectively, in terms of their ability towards hydrophobicity (Figure 10).

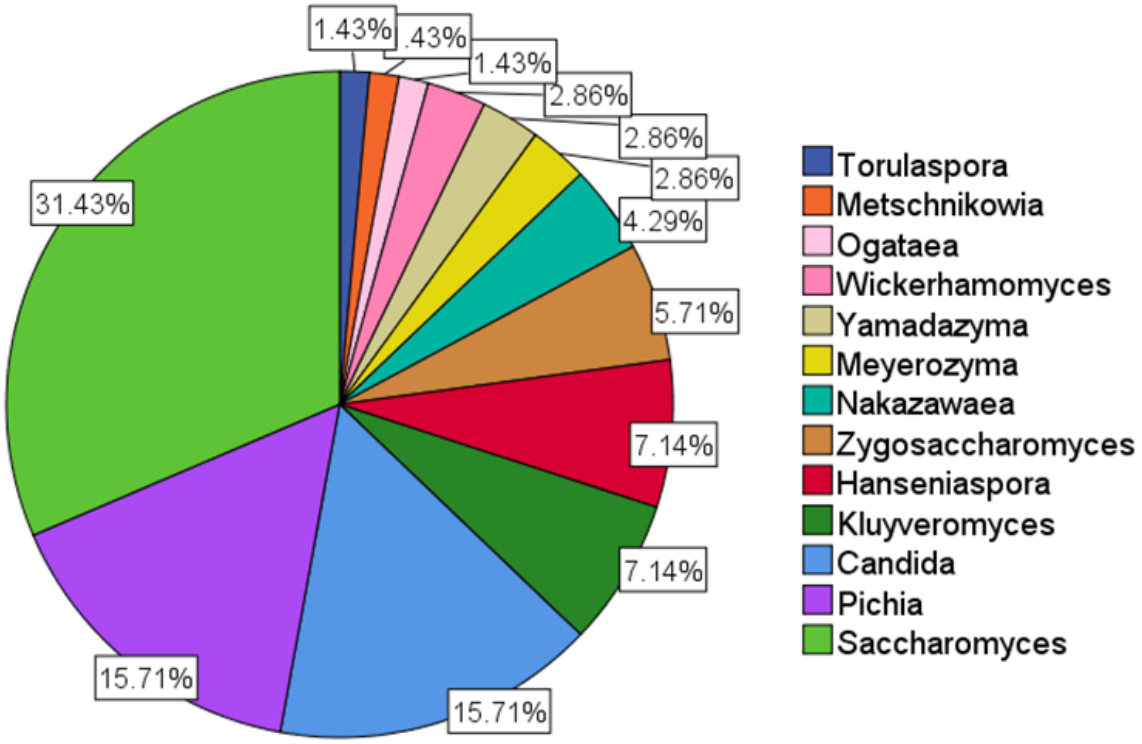

Figure 9: Total number (%) of isolated yeast genera measured as the hydrophobicity ability towards *n*-hexadecane.

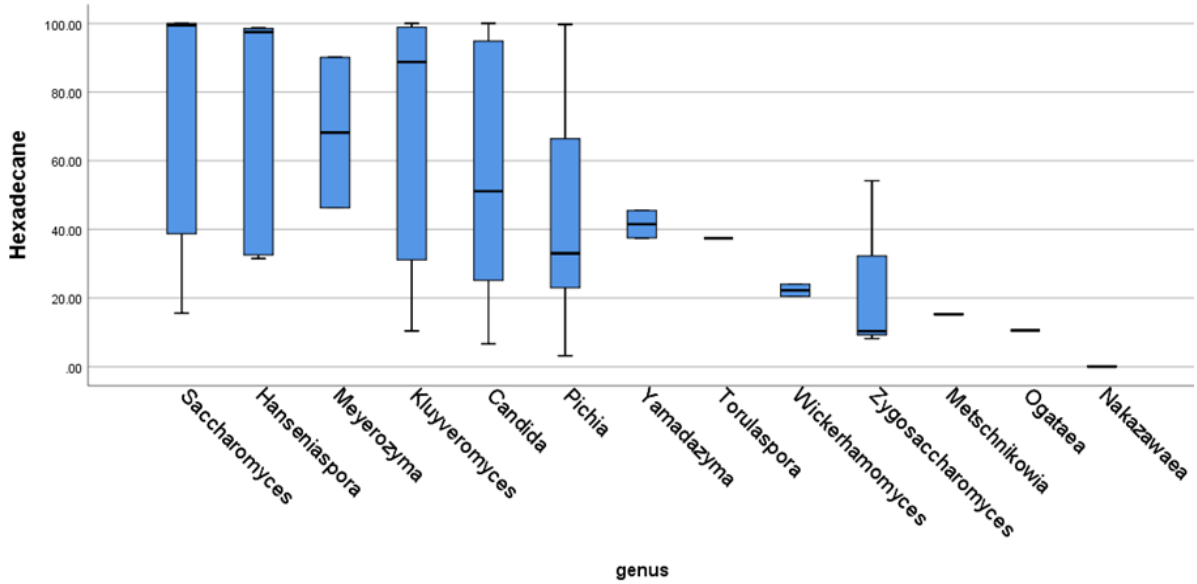

Figure 10: Hydrophobicity (%) towards *n*-hexadecane in descending order according to yeast genera.

2.7.1 Assessment Methods of Hydrophobicity

The technique often used *in vitro* to assess hydrophobicity is measuring microbial interaction with hydrocarbons, such as n-hexadecane. This method is based on the microorganism's affinity to a nonpolar solvent (e.g. *n*-hexadecane) (Goktas et al., 2021b). Initially, strains are resuspended in buffer (e.g., 0.1 M KNO_3 or 0.1 M PBS, pH 7). After measuring $OD_{600\ nm}$ as the initial absorbance, the addition of n-hexadecane, xylene and octane to independent samples is conducted. After incubation at 37°C for 60 min, the absorbance of the aqueous phase is measured (Amorim et al., 2018; Fernandez-Pacheco Rodríguez et al., 2018; Zullo & Ciafardini, 2019).

Gut et al. (2019), Zullo & Ciafardini (2019) and Menezes et al. (2020) used only *n*-hexadecane to examine the capacity of putative yeast probiotics, which may give unsatisfactory results. By contrast, microcell adhesion to the intestinal epithelium is linked to several factors, such as lectins, passive forces, lipoteichoic acid and hydrophobic forces (Romero-Luna et al., 2019; Servin, 2004). Therefore, the ability to be hydrophobic is not determined by the presence of a particular hydrocarbon solvent. For example, Linder (2009) stated that the self-assembly of filamentous fungi refers to hydrophobins and surface-active proteins, whereas Hazen & Hazen (1993) found that the main factor of the hydrophobic feature in *Candida albicans* is the surface fibrils. Thus, the more variety of nonpolar solvents used to evaluate hydrophobicity is, the more accurate are the results.

2.7.2 Mechanisms of Hydrophobicity

Hydrophobicity is a physicochemical characteristic of the microbial cell surface, where a cell surface protein and lipoteichoic acids mediate a nonspecific interaction between microbial and host cells (Hirayama et al., 2012; Willaert, 2018). Lara-Hidalgo et al. (2019) reported that strains with > 40% hydrophobicity are hydrophobic. In this respect, hydrocarbons of the host cell will be bound by those strains and will be shifted from the aqueous to the organic phase of the environment (Kaczorek et al., 2008). Hydrophobicity has been attributed to complex interactions between hydrophobic and hydrophilic (negatively and positively charged) components on the microbial cell surface. To evaluate the cell surface hydrophobicity of newly isolated yeasts, different

hydrocarbons, such as *n*-hexadecane, xylene and octane, should be used to mimic the adhesion efficiency of the intestinal epithelium.

2.8 Insights and Future Recommendations

Studying gastrointestinal and salt tolerance *in vitro* has revealed the possibility of yeast cells surviving under harsh conditions. Thorough knowledge of the mode of action that supports the adaptation of such gastrointestinal and salt stresses can promote yeasts as probiotics. Simulating the required conditions in the food industry and mimicking the biological processes to which yeasts in the human body are exposed are the decisive keys to accurately assess the probiotic properties of potential yeast isolates.

In addition, there is no relationship between yeast availability (Figures 1 and 4) and its survivability (Figures 2, 3 and 5) under stresses and activities (autoaggregation and hydrophobicity), as reviewed by this study. This review also showed that *Pichia* and *Cystofilobasidium* achieved the highest survival rate under GIT stresses, indicating that it is valuable to focus on their molecular and genetic mechanisms to advance to *in vivo* trials to exploit them in the production of functional foods and probiotic dietary supplements. This review included mainly the more robust probiotic properties (GIT and salt tolerance) presented by fermented food compared to unfermented food. In addition, the higher capability of cell surface properties, autoaggregation and hydrophobicity were demonstrated by isolates that achieved higher survivability under GIT stress (positive correlation).

The differences between bacteria and yeasts dictate the necessity to determine the mechanisms adopted by yeast probiotics to overcome extreme conditions. In this context, yeast resistance to bile acids/salts requires further studies. Further investigation is recommended to evaluate potential yeast probiotics in the food industry, such as survivability under heat stress and the ability to produce exopolysaccharides and safety aspects, including antibiotic sensitivity and the absence of virulence genes.

Author Contributions: N.A., writing—original draft, formal analysis; T.M.O., A.N.O., A.A.-N., S.-Q.L., N.P.S., V.A., writing—review and editing; writing—review and editing; M.M.A., conceptualization, writing—original draft, funding, supervising, writing—review and editing, supervision. All authors have read and agreed to the published version of the manuscript.

Funding: This research and APC were funded by the United Arab Emirates University (UAEU), Al-Ain, UAE.

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

Chapter 3: *In vitro* Characterization and Identification of Potential Probiotic Yeasts Isolated from Fermented Dairy and Non-Dairy Food Products

Redrafted from:

Alkalbani, N. S., Osaili, T. M., Al-Nabulsi, A. A., Obaid, R. S., Olaimat, A. N., Liu, S. Q., & Ayyash, M. M. (2022). In Vitro Characterization and Identification of Potential Probiotic Yeasts Isolated from Fermented Dairy and Non-Dairy Food Products. J Fungi (Basel), 8(5), 544. doi:10.3390/jof8050544

3.1 Abstract

This study is about the isolation of yeast from fermented dairy and non-dairy products as well as the characterization of their survival in *in vitro* digestion conditions and tolerance to bile salts. Promising strains were selected to further investigate their probiotic properties, including cell surface properties (autoaggregation, hydrophobicity and coaggregation), physiological properties (adhesion to the HT-29 cell line and cholesterol-lowering), antimicrobial activities, bile salt hydrolysis, exopolysaccharide (EPS) producing capability, heat resistance and resistance to six antibiotics. The selected yeast isolates demonstrated remarkable survivability in an acidic environment. The reduction caused by *in vitro* digestion conditions ranged from 0.7 to 2.1 Log_{10}. Bile salt tolerance increased with the extension in the incubation period, which ranged from 69.2% to 91.1% after 24 h. The ability of the 12 selected isolates to remove cholesterol varied from 41.6% to 96.5%, and all yeast strains exhibited a capability to hydrolyse screened bile salts. All the selected isolates exhibited heat resistance, hydrophobicity, strong coaggregation, autoaggregation after 24 h, robust antimicrobial activity and EPS production. The ability to adhere to the HT-29 cell line was within an average of 6.3 Log_{10} CFU/mL after 2 h. Based on ITS/5.8S ribosomal DNA sequencing, 12 yeast isolates were identified as 1 strain for each *Candida albicans* and *Saccharomyces cerevisiae* and 10 strains for *Pichia kudriavzevii*.

Keywords: Autoaggregation; coaggregation; antimicrobial resistance; probiotics; yeast.

3.2 Introduction

Probiotics are defined as 'live microorganisms that, when administered in adequate amounts, confer a health benefit on the host' (FAO/WHO, 2002). Probiotics contain various microorganisms, including bacteria and yeasts (Perricone et al., 2014). Lactic acid bacteria (LAB) and Bifidobacterial are the main sources of probiotic strains (Doron & Snydman, 2015; Picard et al., 2005), which are widely used as supplements or in food industries. In contrast, to date, only a probiotic yeast, *Saccharomyces cerevisiae* var. *boulardii,* has gained the qualified presumption of safety (QPS) status from the European Food Safety Authority as probiotic supplements (Fernandez-Pacheco Rodríguez et al., 2018). *S. cerevisiae* var. *boulardii* is used in numerous countries to prevent and treat several gastrointestinal disorders (Kelesidis & Pothoulakis, 2012). However, the scientific community is witnessing a significant increase in the number of scientific studies on the isolation, characterization and identification of non-*Saccharomyces* yeasts (e.g., *Pichia*, *Schizosaccharomyces*, *Kluyveromyces*, *Rhodotorula* and *Candida*) and reporting them as promising probiotics (Amorim et al., 2018; Gil-Rodríguez et al., 2015; Lane & Morrissey, 2010; Oliveira et al., 2017).

Yeasts are unicellular eukaryotic microorganisms commonly found in soli, air, water, and food and are of animal and plant origins; they constitute <0.1% of microbiota in the human gut (Czerucka et al., 2007; Foligne et al., 2010). The use of yeasts as probiotics has gained increasing attention within the last few years owing to their high contents of minerals, vitamin B, peptides, proteins and several immunostimulant compounds, such as mannan oligosaccharides, proteases and β-glucans (Arévalo-Villena et al., 2018; Fadda et al., 2017; Gil-Rodríguez et al., 2015). Moreover, yeasts exhibit good resistance to industrial conditions, such as high temperature and lyophilization (Abdel-Rahman et al., 2013; Fleet, 2011a; Joshi & Thorat, 2011).

Currently, yeasts have gained increasing interest in the field of food biotechnology, including their roles in recombinant protein production, alcoholic fermentation and vitamin biosynthesis (Gil-Rodríguez et al., 2015; Hatoum et al., 2012). Furthermore, in the production of bread, beer, table olives, wine, or kefir, yeasts are used as starters (Arroyo-López et al., 2012; Moreira et al., 2011). *Pichia kudriavzevii* and a combination of *S. cerevisiae* var. *boulardii* and inulin are used to produce fermented

cereal-based food and symbiotic yogurt, respectively (Ogunremi et al., 2015; Sarwar et al., 2019). Yeasts are also associated with the maturation of certain cheeses (Binetti et al., 2013). Although yeasts may be a contaminant present in various foods (e.g., fruit juices, chocolate and yoghurt) that could cause food spoilage, many yeasts have been found to exhibit antimicrobial activity against foodborne pathogens and/or spoilage microorganisms (Antunes & Aguiar, 2012; Lowes et al., 2000).

The characterization of new probiotic candidates needs to follow the criteria established by the United Nations/World Health Organization (FAO/WHO) in 2002. The most important among these criteria is tolerance to the gastrointestinal tract (GIT) (Vera-Pingitore et al., 2016) conditions (low pH, digestive enzymes, bile salts and alkaline pH), adhesion to epithelial cells, bile salt hydrolysis (BSH), assimilation of cholesterol in the human intestine and food, antimicrobial activities and antibiotic sensitivity (FAO/WHO, 2002). Furthermore, probiotic candidates should exhibit high-temperature tolerance for industrial purposes and the ability to produce EPS (Silambarasan et al., 2019).

The biofunctional market continuously requires the diversification and application of novel products that provide new probiotic strains with specific functional properties (de Melo Pereira et al., 2018). Probiotic yeasts can provide functional properties that bacterial probiotics cannot. Thus, isolation of new probiotic yeasts is always required to meet the demands of the functional food and beverage market. The present study aimed (1) to isolate novel yeasts from dairy and non-dairy fermented food products, (2) to characterize the potential probiotic attributes of these newly isolated yeasts, including tolerance to the GIT conditions, cell-surface and adhesive properties (autoaggregation, hydrophobicity, coaggregation and HT-29 cell line adhesion), antimicrobial activities, antibiotic sensitivities, heat tolerance, EPS production, ability to remove cholesterol and BSH activity, and (3) to identify the best potential probiotic yeasts using molecular techniques.

3.3 Materials and Methods

3.3.1 Sample Collection

A total of 105 samples of various fermented dairy and non-dairy food products sources free of any food preservatives were collected from different local markets in the United Arab Emirates (UAE). The samples were placed in an icebox and transported to the food microbiology lab of the UAEU for the isolation and characterization of the potential probiotic yeast strains. Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

3.3.2. Isolation of Yeasts

The food samples were serially diluted with 1% peptone water (Neogen, Lansing, MI, USA). The pour-plate technique was employed using Yeast Extract–Peptone–Dextrose (YPD) agar (*Himedia* Laboratories Pvt. Ltd., Nashik, India), and the plates were aerobically incubated at 25°C for 5 days (Binder C 170, Tuttlingen, Germany). Three copies of each colony isolates were subcultured in the YPD broth; subsequently, the stocks were prepared using glycerol (50% v/v) and then stored at −80°C. The potential probiotic characteristics of the yeast isolates were evaluated after two successive activations at 25°C.

3.3.3 Acid tolerance: Preliminary Probiotic Investigation

Acid tolerance of the yeast isolates was evaluated at pH 2.5. A suspension of the tested yeast isolates was prepared in YPD broth and incubated at 25°C for 24 h. The suspension was centrifuged at 5000 × *g* for 10 min, washed with phosphate-buffered saline (PBS) (0.1 M, pH 7) and resuspended in 3 mL YPD broth with the pH adjusted to 2.5 using 1 M HCl. Subsequently, the suspension was distributed in 24-well plates and incubated at 25°C for 24 h. A 1 mL solution of the resuspended yeasts pellets in a YEP broth without pH adjustment (pH 6.7) was considered a control. The growth levels of yeast strains were measured at OD_{600}.

3.3.4 Tolerance to In Vitro Digestion Conditions

In vitro digestion tolerance was evaluated using the method described by Brodkorb et al. (2019). The *in vitro* gastrointestinal INFOGEST 2.0 protocol was applied to the yeast strains. A 2-mL aliquot of the yeast pellet suspension was subjected to *in vitro* digestion, including the oral (amylase 75 U/mL, salivary fluid SSF pH 7.0, 0.3 M $CaCl_2$, 2 min, 37°C), gastric (pepsin 2000 U/mL, RGE 60 U/mL, gastric juice SGF pH 3.0, 0.3 M $CaCl_2$, 120 min, 37°C) and intestinal (pancreatin 100 U/mL, bile 10 mmol/L, duodenal juice SIF pH 7.0, 0.3 M $CaCl_2$, 120 min, 37°C) phases. Continuous shaking at 120 rpm was applied during the *in vitro* digestion process. Serial dilution was performed to directly measure the yeast count before and after the *in vitro* digestion.

3.3.5 Bile Salt Tolerance

The bile salt tolerance of the selected yeast isolates was tested according to AlKalbani et al. (2019). The selected yeasts were tested against 0.3% oxgall, 0.1% cholic acid and 0.1% taurocholic acid, individually, during 0, 6 and 24 h of incubation at 37°C. The growth levels of yeast strains were recorded at OD_{600}.

3.3.6 Cholesterol Removal

According to Alameri et al. (2022), the capability of the selected yeast isolates to remove cholesterol was measured using *o*-phthalaldehyde at 550 nm. The cholesterol removal (%) was expressed as follows:

$$\text{Cholesterol removal (\%)} = \left[\frac{100 - residual\ cholestrol\ at\ each\ incubation\ interval}{100}\right] \times 100$$

3.3.7 Bile Salt Hydrolysis (BSH) Activity

The BSH activities were determined by measuring the amount of amino acids released from conjugated bile salts by yeast strains according to the method described by AlKalbani et al. (2019). The BSH activities were assayed against 6-mM sodium glycocholate, 6-mM sodium taurocholate, or 6-mM conjugated bile salt mixture (glycocholic, glycochenodeoxycholic, taurocholic, taurochenodeoxycholic and taurodeoxycholic acids).

3.3.8 Autoaggregation

Autoaggregation assay of the activated cultures was performed according to the method described in (Bonatsou et al., 2018), and absorbance was measured at 600 nm at the time intervals of 0, 3, 6 and 24 h. The autoaggregation percentage was calculated using the following equation:

$$\text{Auto} - \text{aggregation}(\%) = \left[1 - \frac{At}{A0}\right] \times 100$$

where 'A_t' denotes the absorbance at the time 't', and 'A_0' denotes the absorbance at the time '0'.

3.3.9 Hydrophobicity

Hydrophobicity was evaluated against three different hydrocarbons, *n*-hexadecane, xylene and octane, according to the method described by Fadda et al. (2017). The final absorbance was measured at 600 nm. The hydrophobicity percentage was expressed as follows:

$$\text{Hydrophobicity}(\%) = \left[\frac{A - A0}{A}\right] \times 100$$

where 'A' denotes the initial absorbance at 600 nm, and 'A_0' denotes the final absorbance.

3.3.10 Coaggregation

The coaggregation experiment was conducted according to the method described by Andrade et al. (2021) at 37°C during incubation for 4, 6 and 24 h against four pathogens: *Escherichia coli* 0157:H7 1934, *Staphylococcus aureus* ATCC 25923, *Salmonella* Typhimurium 02–8423 and *Listeria monocytogenes* DSM 20649. The coaggregation percentage was calculated using the following equation:

$$\text{Co} - \text{aggregation}(\%) = \left[\frac{A0 - At}{A0}\right] \times 100$$

where 'A_t' denotes the absorbance at the time 't', and 'A_0' denotes the absorbance at the time '0'.

3.3.11 Antimicrobial Activity

The cell-free supernatant of the activated selected yeast isolates was used to determine the antibacterial activity against four foodborne pathogens: *L. monocytogenes*, *Salmonella* Typhimurium 02-8423, *E. coli* O157:H7 and *S. aureus*. The antimicrobial test was conducted according to the method described by Hossain et al. (2020).

3.3.12 Antibiotic Susceptibility

The resistance of the selected yeast isolates to antibiotics [2-µg clindamycin (CLI), 10-µg ampicillin (AMP), 25-µg trimethoprim-sulfamethoxazole (SXT), 10-µg penicillin (PEN), 30-µg vancomycin (VA) and 15-µg erythromycin (E) (Oxoid; Hampshire, UK)] was evaluated using the YPD agar. This methodology was adapted from Tarique et al. (2022). The interpretative zones of resistant (R), moderately susceptible (MS) and susceptible (S) were defined according to the method described in (Charteris et al., 1998).

3.3.13 Adhesion to the HT-29 Cell Line

To evaluate the adhesion ability of selected yeasts, the activated isolates were washed twice with Dulbecco's phosphate-buffered saline. The adhesion property was tested according to the method described by Hong et al. (2019) and measured in percentage using the following equation:

$$\text{Adhesion ability}(\%) = \left[\frac{At}{A0}\right] \times 100$$

where A_t denotes the number of the adhered cells (log CFU/mL) after incubation, and A_0 denotes the initial cell number (log CFU/mL).

3.3.14 EPS Production

The ability of the selected yeast isolates to produce EPS (−ve/+ve) was measured according to the method described by Angmo et al. (2016), where yeasts cultured overnight were streaked onto the surface of plates containing ruthenium red milk agar (10% w/v skim milk powder, 1% w/v sucrose, 0.08 g/L ruthenium red, 1.5% w/v agar).

3.3.15 Heat Resistance

Heat resistance of the selected yeast isolates was measured according to the method described by Teles Santos et al. (2016) at 60°C for 5 min. Serial dilution was performed to directly measure the yeasts count before and after heat treatment.

3.3.16 Molecular Identification of the Selected Yeast Isolates

A total of 12 yeasts were selected and subjected to PCR amplification of the ITS/5.8S ribosomal DNA. DNA extraction and purification were performed using DNeasy UltraClean Microbial Kit (Qiagen, Carlsbad, CA, USA)) and PCR Kit (BIONEER, Daejeon, Korea) according to the manufacturer's protocols. PCR analysis was conducted as detailed in (Franco-Duarte et al., 2011; Lara-Hidalgo et al., 2019) and according to Amorim et al. (2018) using primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′). Sequencing was performed at the Macrogen sequencing facilities (Macrogen-Korea, Seoul, Korea). Yeasts identification was achieved by comparing the obtained sequences with those available from the NCBI database using the BLAST algorithm. The accession numbers of the selected yeast isolates were obtained by GenBank®. The neighbour-joining method was employed to determine the closest yeast species using the MEGA software version 11 (Saitou & Nei, 1987; Tamura et al., 2013).

3.3.17 Statistical Analysis

To determine whether the variations between yeast isolates had a significant influence on quantitative parameters, one-way ANOVA and Tukey's test were conducted to examine the differences between the mean values at $p < 0.05$. All tests were conducted at least three times.

3.4 Results and Discussion

A total of 105 colonies with different morphological properties were isolated on YPD agar from different food products sold in the local market. The selected yeast isolates were purified and preserved at −80°C in 50% glycerol containing YPD broth.

3.4.1 Preliminary Acid Tolerance

The acid tolerance percentages of 105 isolates at pH 2.5 during 24 h of incubation at 37°C are presented in Appendix B.1 and summarized in Figure 11 (boxplot). The yeasts isolates exhibited various levels of survivability at low pH (0.0% to 100%). A total of 45 yeast isolates that demonstrated noticeable acid tolerance were selected to investigate their tolerance to *in vitro* digestion conditions and bile salt.

The beneficial aspects of probiotics can be exploited if they exhibit resistance to an acidic environment. Thus, acid tolerance is a pivotal factor that allows the candidate probiotic to pass through the gastrointestinal tract (GIT) in a vital and adequate amount and to be used in the food industry. In this study, a low acidic medium pH of 2.5 at 37°C was used as a preliminary indicator for potential probiotic features that could be held in our isolates. Generally, adjustment of yeast cell walls and activation of the cell wall integrity and general stress response pathways are the main strategies that enable the selected probiotic yeasts to resist a strong inorganic acid (Kapteyn et al., 2001; Lucena et al., 2020).

In the present study, high survivability in an acidic medium is preferred. The strains were basically isolated from low-pH environments such as fermented dairy and non-dairy products, where they cohabited with the lactic and/or acetic acid produced by bacteria. In this context, the results of Santos et al. (2012) and Moreira et al. (2013) are consistent with ours. Şanlidere Aloğlu et al. (2016) tested the different yeast species they collected at pH 2.5 according to our acid tolerance conditions.

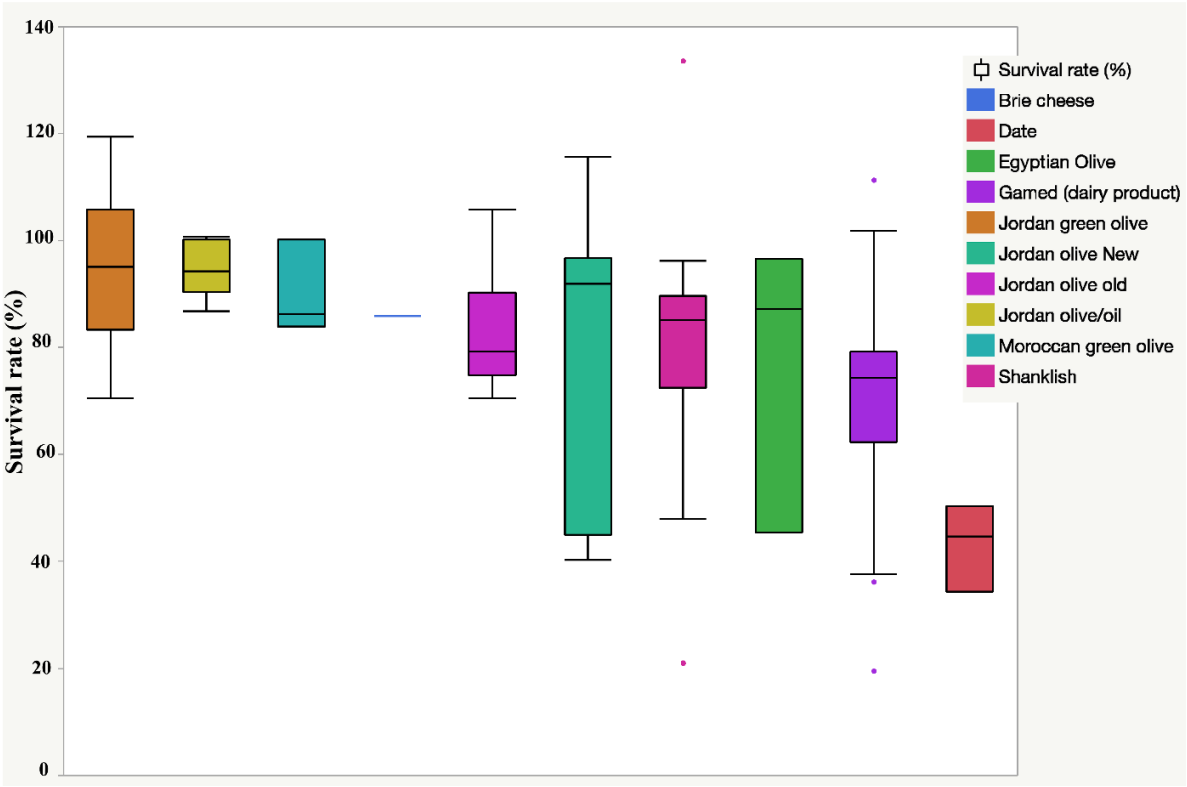

Figure 11: Boxplot summarizing the survival rate (%) of the 105 yeast isolates under pH 2.5 for 2 h at 37°C.
Bullets represent outliners.

3.4.2 Tolerance to In Vitro Digestion Conditions and Bile Salts

Table 1 presents the survival rates of potential yeast probiotics before and after being subjected to *in vitro* digestion with simulated fluids and bile stress against oxgall, cholic acid and taurocholic acid at different concentrations. The growth of all yeast isolates decreased ($p < 0.05$) under *in vitro* digestion conditions. The yeasts' count reduction after *in vitro* digestion ranged from ~ 0.7 to 2.1 Logs. In general, isolates O63, SH45, SH40, O12, O26, SH46 and SH55 exhibited the highest resistance to *in vitro* digestion conditions. On the other hand, the yeast isolates demonstrated remarkable resistance to oxgall compared with cholic and taurocholic acids. The bile salt tolerance of the yeast isolates increased with the extension in the incubation period, which ranged from 43.8% to 87.9%, 17.4% to 85.7% and 68.4% to 86.7% after 6 h and from 48.9% to 90.5%, 26.5% to 89.5% and 69.2% to 91.1% after 24 h. Overall, isolates SH104, SH105, SH 96, G1, SH46, O12 and O24, among others, exhibited high bile resistance. Twelve isolates with high survivability in *in vitro* digestion conditions were selected according

to their varying isolated sources for subsequent investigations. These isolates were G1, O12, O13, O18, O21, O26, O36, O63, O66, SH40, SH45 and SH55.

Table 1: *In vitro* digestion conditions and bile salt tolerances for 45 potential probiotic yeast isolates.

Isolate	Tolerance to GIT			Bile salt tolerances (%)					
				6 h			24 h		
	Before	After	Log Reduction	0.3 CA	1.0 TA	1.0 OX	0.3 CA	1.0 TA	1.0 OX
G.1	7.3±0.01	5.5±0.03	1.8	54.2	36.9	74.1	68.3	81.4	89.1
G.2	7.5±0.09	5.4±0.02	2.1	53.7	36.1	74.8	66.7	73.5	88.1
G.3	7.6±0.24	6.2±0.12	1.4	71.6	52.5	70.7	84.3	82.1	83.3
G.6	7.4±0.09	6.3±0.11	1.1	73.8	61.6	77.2	78.9	81.1	87.5
G.7	7.4±0.13	6.1±0.12	1.3	66.4	68.6	79.7	80.8	83.4	88.0
G.8	7.5±0.11	6.1±0.06	1.4	71.0	81.7	79.6	83.8	85.6	88.3
G.9	7.5±0.07	6.2±0.10	1.3	67.8	76.8	80.0	79.9	81.5	84.5
G.10	7.5±0.02	6.2±0.02	1.4	70.5	64.7	80.8	83.0	80.7	87.6
O.12	7.3±0.06	6.3±0.04	0.9	80.5	78.9	81.0	87.4	84.1	87.4
O.13	7.5±0.06	6.2±0.03	1.3	72.8	68.2	79.2	80.5	81.1	87.1
O.18	7.5±0.01	6.3±0.02	1.2	67.0	80.0	84.8	79.9	81.4	88.7
O.19	7.4±0.04	6.1±0.03	1.3	69.0	45.9	85.8	78.2	58.0	86.2
O.20	7.5±0.02	6.3±0.01	1.2	79.8	42.4	80.6	86.1	81.5	87.1
O.21	7.6±0.19	6.4±0.20	1.3	82.2	59.2	86.3	86.9	80.7	89.1
O.22	7.5±0.04	6.3±0.05	1.2	82.9	76.7	84.2	87.5	79.4	87.9
O.23	7.4±0.08	6.2±0.03	1.2	73.7	67.8	83.8	79.8	75.6	88.0
O.24	7.5±0.03	6.5±0.09	1.0	84.5	70.3	86.7	87.9	83.7	91.1
O.26	7.4±0.06	6.2±0.09	1.2	80.3	69.5	82.0	83.4	77.9	88.2
O.30	7.5±0.01	6.3±0.10	1.2	67.0	61.6	79.7	78.7	75.4	86.0
O.33	7.4±0.03	6.4±0.06	0.9	73.9	62.7	80.5	83.1	69.9	83.9
O.36	7.4±0.05	6.2±0.01	1.3	84.3	79.2	84.3	87.7	85.7	89.8
SH.40	7.4±0.08	6.6±0.09	0.9	73.7	63.4	81.3	81.4	77.9	86.9
SH.45	7.1±0.02	6.1±0.04	1.0	65.1	62.7	84.0	84.6	74.4	86.1
SH.46	7.2±0.10	6.3±0.11	0.9	70.5	63.4	82.2	85.0	76.6	90.7
SH.55	7.0±0.24	6.0±0.16	1.0	73.2	68.2	72.7	86.0	80.9	84.4
O.63	7.1±0.12	6.4±0.08	0.7	64.2	64.8	81.4	66.9	65.9	86.4
O.65	7.2±0.00	6.3±0.04	1.0	68.0	62.7	81.4	77.1	75.1	86.7
G.69	7.3±0.06	6.3±0.06	1.0	43.8	17.4	68.6	48.9	26.5	69.8
O.66	7.2±0.15	5.4±0.07	1.8	53.1	51.7	69.2	57.5	66.0	69.9
G.75	7.4±0.20	5.9±0.11	1.5	52.5	57.0	69.4	57.5	61.3	70.3
G.71	7.5±0.13	6.2±0.16	1.3	76.4	70.4	72.7	86.6	82.7	83.3
G.77	7.2±0.17	5.8±0.12	1.4	78.5	75.7	76.6	87.2	87.3	85.6
G.78	7.1±0.06	6.1±0.04	1.0	81.7	72.7	77.3	88.1	85.5	86.1
G.80	7.2±0.21	5.9±0.10	1.3	67.2	78.0	76.5	80.3	84.3	86.4
G.82	7.4±0.09	5.9±0.06	1.5	78.1	70.1	75.5	85.9	81.1	84.8
G.84	7.2±0.08	5.9±0.04	1.3	78.9	70.7	72.0	87.8	80.8	86.0
SH.96	7.4±0.17	5.8±0.14	1.6	86.8	84.8	75.7	89.7	89.2	90.1
SH.97	7.4±0.08	6.2±0.04	1.3	85.9	84.8	78.1	90.2	89.3	89.8
SH.98	7.2±0.23	5.7±0.09	1.5	87.3	82.4	71.9	89.5	88.8	81.9
SH.99	7.3±0.32	6.3±0.27	1.0	84.9	77.3	68.4	89.6	89.0	69.2
SH.100	7.4±0.13	5.9±0.18	1.5	84.9	83.0	81.1	89.8	89.1	90.3
SH.102	7.3±0.13	5.6±0.07	1.7	83.9	85.7	73.3	89.4	88.7	89.4
SH.103	7.1±0.19	5.5±0.17	1.6	87.9	81.7	79.9	90.8	89.5	90.3
SH.104	7.4±0.30	6.2±0.22	1.2	86.6	79.8	80.0	90.5	89.4	90.6
SH.105	7.4±0.14	6.1±0.08	1.3	86.3	70.5	74.8	89.4	87.8	89.4

Values are expressed as mean ± standard deviation of triplicates.
CA, cholic acid; OX, oxgall; TA, taurocholic acid. Stimulated gastrointestinal tract by INFOGEST.

A probiotic candidate must exhibit high survivability in stressful conditions that it will inevitably face inside the human GIT to exert its functionality. At the start of the digestion process, the potential probiotics should demonstrate tolerance to the amylase present in the oral cavity. After ingestion, the potential probiotics must resist several harsh conditions in the stomach, e.g., presence of low pH, gastric fluid and pepsin (Uymaz Tezel et al., 2021). Next, the probiotic cells must exhibit resistance to the small intestine conditions, such as the presence of pancreatin, bile salts and alkaline stress (de Melo Pereira et al., 2018). Moreover, tolerance to mild heat shock is necessary for the survivability of probiotic strains. The probiotic candidate has to retain its viability and functionality at the internal temperature of the human body (37°C) because 28–30°C is mostly the optimal temperature for yeasts growth (Walker, 2009).

Consequently, the potential probiotic should exhibit low reduction in viability after being subjected to *in vitro* digestion (Ayyash et al., 2021b). Generally, the yeasts probiotic tolerance mechanism to the GIT conditions depends on the species/strain. Bile salts possess antimicrobial activity that could suppress any microorganism, including yeasts. Thus, for microorganisms to be classified as probiotics, they need to resist bile salts. The bile salt resistance of *S. cerevisiae* could be attributed to an increase in its lipid content after being exposed to bile salts and low pH. These lipids contents probably act as a protective agent against bile salt stress (Palma et al., 2015; Zamith-Miranda et al., 2016).

In the light of our results, the resistances of all isolates to the GIT conditions and bile salts are remarkably different depending on the species/strain specificity. Other works yielded promising findings for *P. kudriavzevii* (Greppi et al., 2017) and *S. boulardii* var. *boulardii* strains (Goktas et al., 2021a), which tolerated simulated GIT juices, isolated from fermented cereal foods and commercial food supplements. In agreement with our findings, Chen et al. (2010a), Menezes et al. (2020) and Amorim et al. (2018) proved the capability of different yeast strains isolated from a variety of food sources to tolerate bile salt.

3.4.3 Cholesterol Removal and Bile Salt Hydrolysis (BSH)

Table 2 presents the cholesterol removal and BSH activities of 12 yeast strains. All 12 yeast strains were capable of effectively removing cholesterol from YPD media. Table 2 demonstrates that the cholesterol removal ability significantly differed among the yeast strains, which varied from 41.6% to 96.5%. Strains O21, O26, SH55 and O13 exhibited a higher ability to remove cholesterol compared with the other investigated yeast strains. Regarding BSH, all yeast strains exhibited the capability to hydrolyse screened bile salts forming free cholic acid. This capability ranged from 3.48 to 4.62, 3.40 to 4.01 and 3.56 to 4.77 U/mg for sodium glycocholate, sodium taurocholate and mixture of bile salts, respectively. Strains O12, O26 and O66 demonstrated higher BSH activities than the other investigated yeast strains (Table 2).

Table 2: Cholesterol removal (%) and bile salt hydrolase (BSH) activities (specific activity, U/mg) of 12 potential probiotic yeasts.

Isolate	CR (%)	BSH					
		Na-SG	SA	Na-TA	SA	Bile salt mixture	SA
G1	47.98±7.55^{ab}	1.79±0.05^{abc}	3.70	1.83±0.07^{bc}	3.79	1.72±0.05^{a}	3.56
O12	50.16±8.68^{ab}	1.80±0.07^{bc}	3.68	1.72±0.07^{ab}	3.52	1.84±0.07^{bc}	3.77
O13	71.96±5.20^{d}	2.13±0.10^{e}	4.46	1.88±0.04^{c}	3.93	1.73±0.07^{a}	3.62
O18	62.31±2.35^{cd}	1.90±0.06^{d}	3.85	1.72±0.04^{ab}	3.49	2.11±0.08^{d}	4.27
O21	95.02±1.43^{e}	1.87±0.03^{cd}	4.01	1.70±0.06^{a}	3.65	2.22±0.05^{e}	4.77
O26	91.59±2.47^{e}	2.17±0.03^{ef}	4.55	1.91±0.02^{c}	4.01	2.26±0.04^{e}	4.73
O36	53.58±1.08^{bc}	1.89±0.02^{d}	3.95	1.82±0.05^{abc}	3.81	1.92±0.05^{c}	4.02
O63	47.98±1.95^{ab}	1.74±0.04^{ab}	3.57	1.71±0.05^{ab}	3.50	1.89±0.04^{bc}	3.87
O66	65.42±2.80^{cd}	1.94±0.04^{d}	4.04	1.88±0.02^{c}	3.90	2.04±0.05^{d}	4.25
SH40	39.56±2.86^{a}	1.76±0.02^{ab}	3.48	1.71±0.02^{ab}	3.40	1.81±0.02^{ab}	3.59
SH45	59.81±1.87^{bc}	1.71±0.05^{a}	3.48	1.71±0.02^{ab}	3.48	1.90±0.09^{bc}	3.86
SH55	91.90±2.35^{e}	2.23±0.03^{f}	4.62	1.83±0.03^{bc}	3.79	1.86±0.07^{bc}	3.84

Values are expressed as mean ± standard deviation of triplicates.
Na-SG, sodium glycocholate (6 mM); Na-TA, sodium taurocholate (6 mM); bile salt mixture (6 mM; glycocholic acid, glycochenodeoxycholic acid, taurocholic acid, taurochenodeoxycholic acid, taurodeoxycholic acid); SA, specific activity (U/mg). $^{a–f}$ Means in same column with different lowercase letters differed significantly ($p < 0.05$).

Cholesterol removal is one of the desirable features of probiotics. In the current study, the investigated isolates exhibited cholesterol reduction capability and BSH activities. Cholesterol assimilation by a probiotic microorganism has been attributed to four main mechanisms, namely, attachment to the cell wall, reduction of cholesterol to coprostanol, incorporation of the cholesterol in the cell wall and disruption of the cholesterol micelles by BSH (Ayyash et al., 2018; Ishimwe et al., 2015). Our findings on the cholesterol-lowering ability of the isolated yeasts are superior to those reported in (Chen et al., 2010b; Porru et al., 2018; Şanlidere Aloğlu et al., 2016; Zullo & Ciafardini, 2019).

Probiotics possess BSH activities to act as bile salt detoxifiers and promote competition in the microbial communities within the small intestine (Allain et al., 2018; Ruiz et al., 2013). The ability of probiotic strains to resist the toxicity of conjugated bile salts present in the duodenum is associated with their BSH activity. In agreement with our results, Fadda et al. (2017) and Şanlidere Aloğlu et al. (2016) reported several *yeast* strains isolated from foods exhibiting BSH activity.

3.4.4 Autoaggregation and Hydrophobicity

Table 3 presents the autoaggregation (%) during 24 h of incubation at 37°C and hydrophobicity (%) against hexadecane, xylene and octane. The 12 yeast isolates exhibited a significant percentage of autoaggregation ranging from 37.6% to 66%, 44.5% to 84.0% and 50.7% to 85.8% during 3, 6 and 24 h of incubation, respectively. In general, the autoaggregation percentages increased with the increase in the incubation period. After 24 h, isolates SH45, O36, O26, O66, O23, O28 and O21 showed a higher autoaggregation ability than the other screened isolates. Table 3 demonstrates that the hydrophobicity of the 12 isolates to hexadecane and octane was higher than to xylene. The hydrophobicity percentages ranged from 23% to 50.4%, 28.2% to 46.5% and 4.3% to 42.5% for hexane, octane, and xylene, respectively (Table 3). Isolates SH40, O36, O40, O36, O12, O21 and O26 presented higher hydrophobicity than the other evaluated isolates.

Table 3: Autoaggregation (%) and hydrophobicity (%) of 12 potential probiotic yeast isolates.

Isolate	Autoaggregation (%)			Hydrophobicity (%)		
	3 h	6 h	24 h	n-Hexane	Octane	Xylene
G1	42.3±0.28^{b}	56.7±1.13^{b}	69.8±1.57^{b}	36.8±3.04^{bcde}	42.31±1.85^{fg}	6.51±2.21^{a}
O12	58.9±0.55^{cd}	73.6±0.60^{c}	80.7±0.32^{c}	32.6±5.71^{abcd}	36.7±5.24^{cde}	25.16±2.55^{bcde}
O13	60.7±0.44^{de}	75.8±1.14^{c}	83.2±0.75^{de}	30.1±1.15^{ab}	40.65±0.86^{efg}	13.08±7.56^{ab}
O18	64.1±0.51^{fg}	78.4±0.46^{c}	82.8±1.00^{d}	31.5±1.95^{abc}	43.46±3.02^{g}	24.86±4.20^{bcde}
O21	65.1±0.21^{gh}	77.0±2.41^{c}	83.7±0.13^{de}	41.9±1.45^{de}	35.21±1.07^{bcd}	20.73±2.72^{abcd}
O26	65.6±0.35^{gh}	77.5±0.75^{c}	84.4±1.11^{def}	37.6±2.76^{bcde}	34.46±1.47^{abcd}	37.72±3.31^{e}
O36	59.2±2.49^{cd}	75.0±2.64^{c}	84.8±1.01^{ef}	42.9±1.11^{e}	42.27±2.68^{fg}	15.62±2.98^{abc}
O63	37.7±0.75^{a}	47.0±2.53^{a}	51.0±0.28^{a}	30.7±2.36^{ab}	30.67±1.27^{a}	23.71±4.37^{bcde}
O66	62.6±0.34^{ef}	77.8±0.22^{c}	82.9±1.15^{de}	24.9±1.12^{a}	31.84±3.67^{ab}	18.03±1.78^{abc}
SH40	42.8±1.38^{b}	57.2±0.49^{b}	83.7±0.05^{de}	41.2±3.61^{cde}	44.98±1.57^{g}	29.55±8.17^{cde}
SH45	66.6±0.31^{h}	75.3±4.86^{c}	86.1±0.55^{f}	33.6±1.84^{abcde}	38.51±3.84^{def}	21.19±3.46^{abcd}
SH55	58.5±0.06^{c}	71.3±0.51^{c}	80.3±1.43^{c}	28.3±1.72^{ab}	32.85±1.14^{abc}	33.11±9.87^{de}

Values are expressed as mean ± standard deviation of triplicates.
$^{a–h}$ Means in same column with different lowercase letters differed significantly ($p < 0.05$).

The adherence of microorganisms to epithelial cells in the human intestine can be deduced by their cell surface properties, represented by testing the autoaggregation capability and hydrophobic properties of probiotic candidates (Merchán et al., 2020). A higher aggregation capacity provides high cell intensity involving the adhesion mechanism, whereas a robust hydrophobic property facilitates the attachment between the microbe and epithelial cells (de Melo Pereira et al., 2018). In the present study, the yeast strains exhibited significant percentages of autoaggregation and hydrophobicity to the investigated hydrocarbons. However, there were remarkable distinctions among the screened isolates, which may be attributed to the difference in the hydrophilic and hydrophobic regions in the cell wall of the microbial isolates (Abushelaibi et al., 2017). In addition, Verstrepen & Klis (2006) reported that the differential expression of the adhesin genes in the yeast allows them to rapidly adjust their adhesive properties to a specific environment. It is noteworthy that the size of the yeasts cell are 10 times larger than that of bacteria (Czerucka et al., 2007). Therefore, an individual yeasts cell requires a larger area to adhere to the human intestinal cell surface (Kumura et al., 2004).

In this work, the increasing trend of autoaggregation throughout 24 h is consistent with the findings of Bonatsou et al. (2018), whereas both the autoaggregation and hydrophobicity results are superior to those reported by Zullo & Ciafardini (2019). The drawback of the latter study (Zullo & Ciafardini, 2019) was that the hydrophobicity of yeasts was examined against one hydrocarbon (hexadecane). Moreover, the autoaggregation capacity of the yeasts was tested for only 4 h.

3.4.5 Coaggregation and Antimicrobial Activity

The coaggregation percentages of 12 yeast strains in the presence of *E. coli O157:H7, Salmonella Typhimurium, L. monocytogenes* and *S. aureus* at 3, 6 and 24 h of incubation at 37°C and antimicrobial activities against the same four pathogens are presented in Table 4. The coaggregation capability increased ($P < 0.05$) during the incubation period of 3 to 24 h at 37°C, particularly with *Salmonella Typhimurium*. However, from another view, the yeast isolates had the highest coaggregation percentages with *L. monocytogenes* than the other three pathogens during the incubation period. Overall, isolates O12, O21, O26, O66 and SH45 had a higher coaggregation

percentage than the other investigated strains. The antimicrobial activity presented in Table 4 ranges from 0.1 to > 2.0-mm zone. Interestingly, all yeast strains exhibited substantial inhibition activities against all four pathogens, except the G1, O26 and O13 isolates.

Table 4: Coaggregation (%) and antimicrobial activity of 12 potential probiotic yeast isolates against 4 foodborne pathogens.

Isolate	*S.* Typhimurium				*E. coli* O157:H7				*S. aureus*				*L. monocytogenes*			
	3 h	6 h	24 h	A.M	3 h	6 h	24 h	A.M	3 h	6 h	24 h	A.M	3 h	6 h	24 h	A.M
G1	12.2±1.53^{b}	23.9±0.46^{a}	42.7±1.79^{a}	+++	12.8±0.55^{h}	16.5±0.97^{a}	38.3±0.62^{a}	+++	18.0±0.82^{f}	26.8±0.97^{f}	48.3±0.98^{e}	+	23.8±0.65^{a}	33.7±0.12^{a}	52.1±0.20^{a}	+++
O12	17.3±0.01^{c}	46.7±0.54^{cd}	59.7±1.18^{cd}	+++	46.1±1.04^{a}	51.3±0.07^{f}	64.2±0.08^{ij}	+++	23.0±0.62^{d}	48.7±0.33^{a}	62.4±1.76^{a}	+++	38.5±0.45^{d}	45.9±1.15^{b}	61.9±0.83^{c}	+++
O13	25.8±0.61^{e}	52.9±1.33^{ef}	65.3±0.15^{e}	+	38.9±0.26^{cd}	46.2±0.09^{def}	62.0±0.85^{gh}	+++	26.8±0.78^{c}	37.4±0.46^{cd}	53.8±0.46^{cd}	+	28.9±0.21^{b}	40.1±0.59^{ab}	57.2±0.14^{b}	+++
O18	35.3±0.93^{g}	58.6±1.55^{g}	65.4±2.67^{e}	+++	37.2±1.04^{d}	47.4±2.45^{def}	59.9±0.86^{ef}	+++	21.9±0.08^{de}	31.9±1.16^{e}	48.3±1.06^{e}	+++	49.9±1.08^{f}	60.2±0.95^{d}	68.9±2.07^{d}	+++
O21	21.5±0.37^{d}	50.1±0.42^{de}	62.2±0.59^{de}	+++	43.5±1.05^{ab}	51.7±0.83^{f}	65.4±0.45^{j}	+++	19.5±0.78^{ef}	37.6±0.10^{cd}	49.8±1.50^{de}	+++	47.1±0.26^{e}	57.6±2.25^{cd}	69.6±1.19^{d}	+++
O26	21.9±0.84^{d}	50.8±1.08^{de}	62.6±1.03^{de}	+++	41.1±0.73^{bc}	50.3±0.38^{f}	63.1±0.11^{hi}	+++	21.0±0.70^{def}	46.8±1.94^{ab}	60.1±0.91^{ab}	+	45.7±1.09^{e}	52.9±0.30^{c}	66.8±0.62^{d}	+++
O36	12.0±0.95^{b}	43.4±1.25^{bc}	57.4±0.06^{bcd}	+++	22.6±0.54^{f}	35.0±0.42^{c}	52.1±1.21^{c}	+++	14.1±0.12^{g}	27.3±0.49^{f}	48.5±1.28^{e}	+++	48.0±0.10^{ef}	55.2±3.36^{cd}	68.5±1.12^{d}	+++
O63	36.0±2.60^{gh}	42.6±0.16^{bc}	54.2±0.36^{b}	+++	17.2±1.15^{g}	26.9±1.09^{b}	44.4±0.72^{b}	+++	32.7±0.68^{b}	37.6±1.66^{cd}	52.7±0.86^{cde}	+++	33.0±0.45^{c}	40.0±0.93^{ab}	53.4±3.19^{ab}	+++
O66	31.6±0.63^{f}	56.0±0.39^{fg}	65.0±1.23^{e}	+++	32.4±0.88^{e}	42.9±3.03^{de}	58.5±1.68^{e}	+++	40.6±1.41^{a}	48.0±0.52^{a}	62.1±0.82^{a}	+++	52.5±0.30^{g}	60.1±2.06^{d}	70.0±0.42^{d}	+++
SH40	37.9±0.00^{h}	55.8±1.41^{fg}	67.3±2.02^{e}	+++	33.5±0.24^{e}	41.9±1.92^{d}	54.6±1.45^{d}	+++	23.1±0.56^{d}	35.3±0.68^{de}	33.3±1.00^{f}	+++	55.5±0.71^{h}	59.7±2.94^{d}	69.3±2.19^{d}	+++
SH45	9.30±0.31^{a}	40.2±0.04^{b}	55.4±1.49^{bc}	+++	30.4±0.49^{e}	44.3±1.41^{de}	60.4±0.97^{fg}	+++	34.0±1.06^{b}	43.2±0.23^{b}	58.6±1.71^{ab}	+++	47.0±0.20^{e}	59.2±1.07^{cd}	69.8±2.24^{d}	+++
SH55	18.8±0.36^{c}	48.1±1.93^{d}	58.3±0.04^{bcd}	+++	39.4±0.80^{cd}	47.9±0.66^{ef}	61.6±0.07^{fgh}	+++	34.6±1.61^{b}	39.2±1.07^{c}	56.4±0.39^{bc}	+++	29.0±0.97^{b}	37.5±0.42^{a}	57.5±4.19^{b}	+++

Values are expressed as mean ± standard error of triplicates.

A.M: antimicrobial activity.

$^{a–j}$ Means in same column with different lowercase letters differed significantly ($p < 0.05$).

The capability of the probiotics to coaggregate with the foodborne pathogens and their potential to displace these pathogens are critical for protection against enteric infections (Burns et al., 2011). Yeast probiotics prevent the pathogens from adhering to the intestinal epithelial cells by adhering to them instead and then cocurating their binding sites (Andrade et al., 2021). Generally, probiotics adapt a coaggregation behaviour to form a competitive microenvironment surrounding the pathogen (Pericolini et al., 2017). The suggested coaggregation mechanism between yeasts and bacterial pathogens has been proposed by Millsap et al. (1998), who stated that particular bacterial pathogens have binding molecules on their surfaces that allow them to bind to mannose residues on the yeasts cell surface. In addition to mannans, glucans and chitin, which are the main components of the yeasts cell wall, all may be associated with yeasts coaggregation with pathogenic bacteria (Hatoum et al., 2012). Several studies have also confirmed particular pathogenic bacteria bound to *S. boulardii*, *Debaryomyces hansenii* and *Yarrowia lipolytica* (Caruffo et al., 2016; Gedek, 1999; Pontier-Bres et al., 2014). Our strains exhibited an intermediate coaggregation ability. However, the higher coaggregation results for all four investigated pathogens are superior to those for *Kluyveromyces lactis* and *Torulaspora delbrueckii* toward the same four pathogens (Andrade et al., 2021).

The antimicrobial activity of probiotics is an essential characteristic represented by antimicrobial compound production, competing exclusion of the pathogens and promotion of the intestinal barrier function (Fijan et al., 2016). Several mechanisms have been postulated for antagonistic yeasts against pathogenic bacteria, including (1) competition for nutrients and space between yeast probiotic and microbial pathogens; (2) pH changes in the environment due to the metabolic activity of the yeasts, leading to stressful conditions for the pathogens; (3) production of high-concentration ethanol; and (4) release of antibacterial substances and secretion of antimicrobial compounds, such as mycocins or killer toxins (Golubev, 2006; Hatoum et al., 2012; Pais et al., 2020; Young & Yagiu, 1978). In this work, *P. kudriavzevii* represents the majority of the tested isolates, and it belongs to the *Pichia* genus, which was deeply reviewed as a producer of killer toxins that can inhibit particular pathogens by Belda et al. (2017).

Our antimicrobial activity results are in contrast to those of Amorim et al. (2018) because no antimicrobial activity was exhibited by their tested yeast isolates (*Candida lusitaniae* and *Meyerozyma caribbica*). However, the results obtained by Hossain et al. (2020) coincide with the current study. Furthermore, the results of the current study indicated that the differences in the antimicrobial activity among the yeast isolates might be attributed to species and strain specificity.

3.4.6 Antibiotic Susceptibility and Attachment to the HT-29 Cell Line

The antibiotic resistance of 12 yeast strains against 6 antibiotics is presented in Table 5. All yeast strains were sensitive or moderately sensitive to all the investigated antibiotics, except strains G1, O12, O13 and O26. Table 5 demonstrates that the yeast strains were more susceptible to erythromycin and clindamycin. Regarding the HT-29 cell line adhesion, the range of yeasts' adhesion to the HT-29 cell line was 5.97–6.99 Log_{10} CFU/mL (Table 5). Generally, isolates G1, O12, O13 and SH45 had the highest ability for HT-29 cell line attachment.

The antibiotic resistance of probiotics is deemed a safety concern because there is a chance of antimicrobial-resistance gene horizontally transmitting to the pathogens (de Melo Pereira et al., 2018). Therefore, potential probiotics with antibiotic sensitivity are desirable. In our work, eight strains were found to be susceptible or moderately susceptible to various commercial antibiotics. Our results are almost in line with those of Amorim et al. (2018) and Hossain et al. (2020), who isolated yeast species from pineapple and soya paste, respectively. The minor disparities between our study and others can be attributed to strain and species variations.

The capability to adhere to the intestinal epithelium is one of the primary criteria for probiotic candidate selection. This capability is considered a pre-condition to exclude enteropathogenic bacteria or promote host immunomodulation (Blum et al., 1999; Isolauri et al., 1999). Expressed proteins located on the surface of the cell walls are associated with microbial adhesion to intestinal epithelial cells (Åvall-Jääskeläinen et al., 2003; Kumura et al., 2004). Generally, the results obtained from the present work showed suitable attachment to the HT-29 cell line. Several studies verified the adhesion abilities of different yeast strains isolated from food sources using the HT-29 cell line (Chen et al., 2010b; Hong et al., 2019; Simões et al., 2021).

Table 5: Antibiotic resistance to 6 different antibiotics.

Isolate	Antibiotic resistance						Attachment to HT-29 Cells
	CLI	AMP	SXT	PEN	VAN	ERY	Log10 CFU
G1	MS	MS	MS	R	MS	S	6.66 ± 0.06^{e}
O12	MS	S	MS	MS	R	S	6.82 ± 0.17^{e}
O13	MS	MS	MS	R	R	MS	6.65 ± 0.06^{e}
O18	S	S	S	S	S	S	6.27 ± 0.06^{bcd}
O21	MS	MS	MS	S	MS	MS	6.00 ± 0.06^{a}
O26	S	R	R	MS	S	S	6.23 ± 0.26^{bcd}
O36	MS	MS	MS	MS	MS	MS	6.15 ± 0.04^{abc}
O63	S	MS	MS	MS	S	S	6.16 ± 0.19^{abc}
O66	MS	MS	MS	MS	MS	MS	6.37 ± 0.04^{cd}
SH40	MS	MS	S	S	MS	MS	6.36 ± 0.17^{cd}
SH45	MS	S	S	S	S	MS	6.41 ± 0.02^{d}
SH55	MS	S	S	MS	S	S	6.06 ± 0.03^{ab}

Values are expressed as mean ± standard deviation of triplicates.

[a] CLI, clindamycin (2 µg); AMP, ampicillin (10 µg); SXT, trimethoprim-sulfamethoxazole (25 µg); PEN, penicillin (10 µg); VAN, vancomycin (30 µg); ERY, erythromycin (15 µg); R, resistant; MS, moderately susceptible; S, Susceptible.

[a–e] Means in same column with different lowercase letters differed significantly ($p < 0.05$).

3.4.7 EPS Production and Heat Resistance

Interestingly, all 12 isolates showed the potential to produce EPS, as presented in Table 6.

Table 6: Exopolysaccharide (EPS) production and heat resistance (Log_{10} CFU/mL) of 12 potential probiotic yeast isolates.

Isolate	EPS production	Heat resistance (Log_{10} CFU/mL)	
		Before	After
G1	+	6.6 ± 0.01^{a}	4.4 ± 0.02^{a}
O12	+	7.5 ± 0.13^{efg}	5.2 ± 0.17^{c}
O13	+	7.7 ± 0.03^{g}	5.3 ± 0.00^{cd}
O18	+	7.3 ± 0.05^{bcd}	5.6 ± 0.06^{f}
O21	+	7.3 ± 0.02^{bcd}	5.5 ± 0.02^{ef}
O26	+	7.3 ± 0.07^{bcd}	5.3 ± 0.07^{cd}
O36	+	7.5 ± 0.00^{def}	5.4 ± 0.03^{cde}
O63	+	7.2 ± 0.06^{bcd}	5.3 ± 0.17^{cde}
O66	+	7.3 ± 0.04^{cde}	4.7 ± 0.10^{b}
SH40	+	7.1 ± 0.02^{b}	5.4 ± 0.02^{def}
SH45	+	7.6 ± 0.07^{fg}	5.3 ± 0.13^{cd}
SH55	+	7.2 ± 0.03^{bc}	4.6 ± 0.24^{ab}

Values are expressed as mean ± standard deviation of triplicates.
$^{a–g}$ Means in same column with different lowercase letters differed significantly ($p < 0.05$).

The EPS production of the yeast isolates was inferred by creating a white ropy mucus on ruthenium red skim milk agar plates. Numerous microorganisms, including yeasts, can produce EPSs, which may vary in their monomer composition, molecular weight and type and degree of branching (Schmid et al., 2016). Therefore, EPSs differ in their functions and applications, which are most related to adhering to, protecting and retaining compounds (Costa et al., 2018). The research group (Rahbar Saadat et al., 2020) had reported EPS production and isolation by yeast, *K. marxianus and P. kudriavzevii*, which were isolated from dairy products. On the other hand, Fekri et al. (2020) revealed that their yeast strains isolated from traditional sourdough, *K. marxianus*, *K. lactis* and *K. aestuarii* produced a higher amount of EPS compared with those of isolated yeasts in the same research (Fekri et al., 2020).

The heat resistance of 12 yeast isolates is presented in Table 6. The growth of all isolates reduced ($p < 0.05$) after they were treated at 60°C for 5 min. The decrease in

yeast growth ranged from 1.7 to 2.6 Log_{10} CFU/mL. Isolates O18, O21, O63 and SH40 presented higher heat resistance compared with other isolates. Heat resistance is a fundamental challenge faced by probiotics when used in the food industry. In the present study, all yeast isolates demonstrated good tolerance to heat. One of the suggested mechanisms for the yeasts to resist extreme heat is the production of trehalose, a sugar produced by a wide variety of microorganisms. The intracellular accumulated trehalose is involved in promoting thermotolerance of the yeasts (Singer & Lindquist, 1998). Several studies have evaluated the heat resistance of yeast probiotics using a method that mainly focuses on testing at only 37°C, which is the internal temperature of the human body (Gil-Rodríguez et al., 2015; Gut et al., 2019; Parafati et al., 2022). The drawback of this method is that it only evaluates the use of probiotics as a supplement, not its use in the food industry, which requires higher temperature. In the studies conducted by Hu et al. (2018) and Hossain et al. (2020), the heat resistance of *S. cerevisiae and S. cerevisiae* var. *boulardii* was tested up to 42°C and 48 °C for 30 min and 72 h, respectively. The isolates in both studies (Hossain et al., 2020; Hu et al., 2018) exhibited a significant reduction in growth rate after heat treatment compared with our isolates. The trend of the heat resistance of *S. cerevisiae* has been reported by Kalyuzhin (2011).

3.4.8 Molecular Identification of Selected Yeast Isolates

A total of 12 potential yeast probiotics were identified using ITS/5.8S ribosomal DNA sequences. Each isolate's name and accession number obtained from GenBank are presented in Table 7. Molecular phylogeny analysis was conducted and a phylogenetic tree constructed to identify yeasts to a species level based on the 1ITS/5.8S ribosomal DNA sequences from evolutionary distances using the neighbour-joining method. The phylogenetic tree of the 12 isolates is presented in Figure 12. The genotyping of *S.cerevisiae,* one of the yeast species included in the current paper, has been widely discussed (Franco-Duarte et al., 2015; Franco-Duarte et al., 2014). One of the most reliable methods used to amplify the genomic sequences is PCR-amplification of inter-delta sequences, where delta elements create the LTR flanking retrotransposons TY1 and TY2 in *S. cerevisiae* (Franco-Duarte et al., 2011). Therefore, in order to distinguish *S.cerevisiae* strain, the use of inter-delta sequencing is recommended.

Table 7: Identification of yeast isolates using ITS/5.8S ribosomal DNA and their accession numbers obtained from GenBank.

Isolate	Microorganism	Accession No	Source
G1	*Candida* sp.	OK441052	Gamed (fermented dried yogurt)
O12	*Pichia kudriavzevii*	OK441055	Jordanian Olive
O13	*Pichia kudriavzevii*	OK441056	Jordanian Olive
O18	*Pichia kudriavzevii*	OK441057	Jordanian Olive in oil
O21	*Pichia kudriavzevii*	OK441060	Jordanian Olive in oil
O26	*Pichia kudriavzevii*	OK441064	Moroccan green olives
O36	*Pichia kudriavzevii*	OK441067	Jordanian green olives
O63	*Pichia* sp	OK441068	Jordanian green olives
O66	*Saccharomyces cerevisiae*	OK441070	Jordanian green olives
SH40	*Pichia kudriavzevii*	OK441071	Shanklish (curdled & dried yogurt)
SH45	*Pichia kudriavzevii*	OK441072	Shanklish (dried yogurt)
SH55	*Pichia kudriavzevii*	OK441073	Shanklish (curdled & dried yogurt)

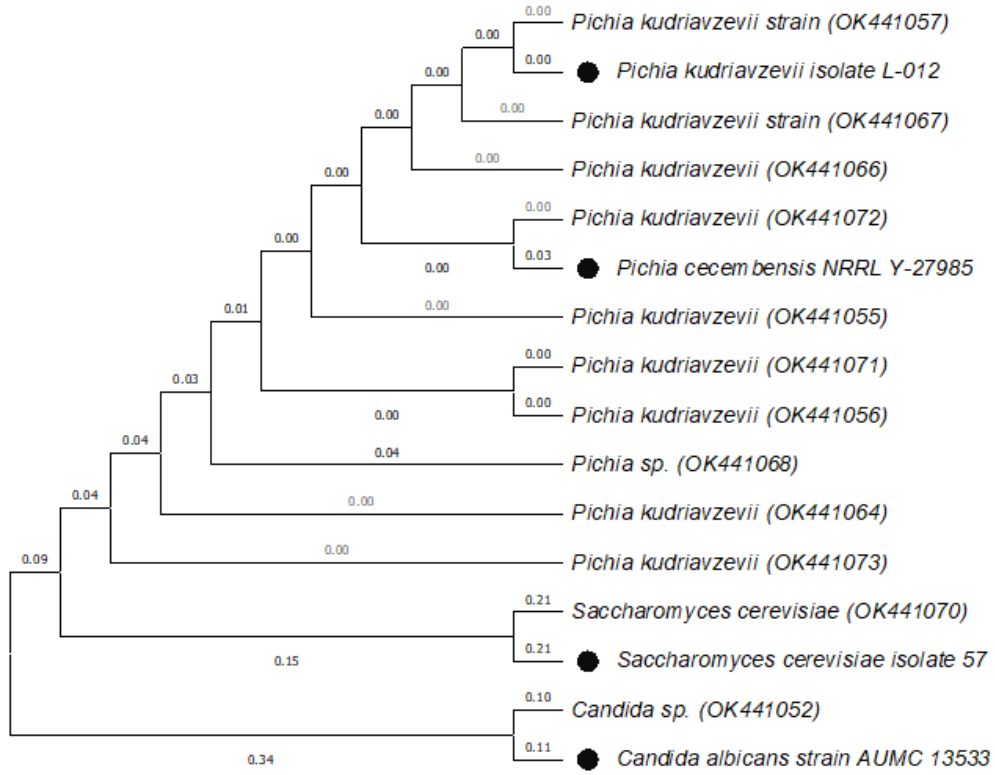

Figure 12: Neighbour-joining phylogenetic tree based on ITS/5.8S ribosomal DNA.
The numbers in parentheses are accession numbers of the identified sequences from the GenBank. The filled circles are the reference strains from NCBI.

3.5 Conclusions

Selected yeast strains from fermented dairy and non-dairy products demonstrated probiotic characteristics. The probiotic yeasts exhibited an excellent survival rate after the *in vitro* digestion, with a 0.7 Log reduction for the highest *in vitro* digestion resistance. The yeast isolates were able to hydrolyse bile salts and significantly reduce cholesterol. The susceptibility of these strains to the tested antibiotics did not present any concerns. The autoaggregation of 12 isolates ranged from 50.7% to 85.8% during 24h of incubation. All those isolates exhibited a higher percentage of hydrophobicity to hexadecane and octane compared with xylene. Generally, the increase in coaggregation percentages during incubation time from 3 h to 24 h was remarkable ($p < 0.05$). The isolates showed significant inhibition activities against the four screened pathogens except G1, O26, and O13 isolates. Overall, the 12 isolates had moderate ability to attach to the HT-29 cell line. The reduction in the growth of 12 isolates after heat treatment ranged from 1.7 to 2.6 LoG_{10} CFU/mL. All the yeast isolates can produce exopolysaccharides (EPS), and isolates SH40 (*Pichia kudriavzevii* OK441071), SH55 (*P. kudriavzevii* OK441073), O63 (*Picha* sp. OK441068) and O66 (*S. cerevisiae* OK441070) have promising probiotic traits, which necessitate further characterization for their use in the food industry.

Author Contributions: N.S.A., writing—original draft, investigation, data curation, formal analysis; T.M.O., A.N.O., A.A.A.-N., S.-Q.L., and R.S.O., writing—review and editing; writing—review and editing; M.M.A., conceptualization, writing—original draft, funding, supervising, writing—review and editing, supervision. All authors have read and agreed to the published version of the manuscript.

Funding: This research and APC were funded by the United Arab Emirates University (UAEU), Al-Ain, United Arab Emirates.

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

Chapter 4: Fermentation of Date Pulp Residues Using *Saccharomyces cerevisiae* and *Pichia kudriavzevii*—Insights into Biological Activities, Phenolic and Volatile Compounds, Untargeted Metabolomics, and Carbohydrate Analysis Post In Vitro Digestion

Redrafted from

Alkalbani, N. S., Alam, M. Z., Al-Nabulsi, A., Osaili, T. M., Olaimat, A., Liu, S. Q., Kamal-Eldin, A., & Ayyash, M. (2023). Fermentation of Date Pulp Residues Using *Saccharomyces cerevisiae* and *Pichia kudriavzevii*—Insights into Biological Activities, Phenolic and Volatile Compounds, Untargeted Metabolomics, and Carbohydrate Analysis Post In Vitro Digestion. Fermentation, 9(6). doi:10.3390/fermentation9060561

4.1 Abstract

Utilizing agricultural byproducts, such as date pulp residues (DPR), can contribute significantly towards achieving sustainable food production. This paper aimed to investigate the chemical properties of DPR and evaluate the health benefits of nonfermented and fermented DPR samples both pre- and post-digestion (bioaccessible portions). Additionally, it aimed to analyze the carbohydrates and untargeted metabolites in the bioaccessible portions. Fermentation of DPR by *Saccharomyces cerevisiae* and *Pichia kudriavzevii* resulted in an abundance of malic acid (over 2400 mg/100 g) and the identification of 42 volatiles, with different degrees of predominance observed in the samples. Twenty phenolics were determined by UPLC in fermented DPR, with (-)-epicatechin, tyrosol, and gallic acid being the most abundant. Bioaccessibility studies revealed that fermented DPR samples retained at least ~44% of α-glucosidase inhibition and exhibited improved α-amylase inhibition compared to nonfermented and undigested samples. In vitro cytotoxicity assays showed a more potent inhibitory effect of fermented DPR against MCF-7 and Caco2 cell lines (average inhibition of 55% and 74.4% for the two types of fermented samples) compared to nonfermented DPR. The untargeted metabolomics analysis identified C5-branched dibasic acid metabolism as the most prominent pathway, with four metabolites identified. Furthermore, the analysis of bioaccessible carbohydrate metabolites in the fermented DPR using LC-QTOF showed the presence of a group of phytochemicals, including three terpenoid metabolites.

Keywords: Date pulp; antioxidant; amylase; glucosidase; ACE-inhibition; cytotoxicity.

4.2 Introduction

The increasing amount of food waste has become a global concern due to its adverse effects, which are not limited to the environment but also extend to the economy and society (Capanoglu & Tomás-Barberán, 2022). According to the Food and Agriculture Organization of the United Nations (FAO) statistics, around 1.3 billion tons of food waste is generated yearly, which equals one-third of the overall food produced for human consumption being wasted or lost (FAO, 2013). Recently, food waste has garnered the scientific community's attention because of its availability and composition (Tropea, 2022). This attention has led to the valorization of food waste through various approaches, where it can be used as a source of nutrient supplements, enzymes, biopolymers, food-grade pigments, and as an efficient substrate for producing novel value-added products (Bilal & Iqbal, 2019; Patel & Shukla, 2017; Sharma et al., 2021).

The date palm (*Phoenix dactylifera* L.) has been extensively cultivated in arid and semiarid regions of the world (Krueger, 2021). The top producers of date fruit globally in 2021, ranked in decreasing order, are Egypt, Saudi Arabia, Iran (Islamic Republic of), Algeria, Iraq, Pakistan, Sudan, Oman, United Arab Emirates, and Tunisia (FAOSTAT, 2021). Production of date fruit was estimated at 9.6 million tons in 2021 (FAOSTAT, 2021). Due to their high nutritional value, date fruits are now being produced, processed, and applied on a larger scale worldwide, corresponding to an increase in the levels of date fruit waste. Annually, around 20% of the date fruit production is lost as a result of over-ripening, inappropriate packaging and storage, contamination, and rough handling (Oladzad et al., 2021). Typically, fresh dates are marketed, while low-quality and immature dates are utilized to produce food products such as syrup, jam, juice, chutney, and jelly (Al-Alawi et al., 2017; Siddiq et al., 2013). The date processing industry generates a significant amount of date residues represented by date pulp residues (DPR), date seeds, and cull date fruits, which are mostly handled as waste (Majzoobi et al., 2020). Improper disposal of these residues can result in severe environmental pollution, health issues, and economic problems (Cheok et al., 2018). Viewed from another

perspective, various scientific studies have reported that the high nutritional content of date processing waste makes it an efficient raw material for producing high-value-added bioproducts (Ahmad et al., 2021; Seyed Reihani & Khosravi-Darani, 2019).

DPR is a solid byproduct of the date syrup/juice production process in the date processing industry (Rezazadeh Bari et al., 2010). About 17–28% of the DPR produced daily is generated by the date syrup industry, some of which is utilized as animal feed while the rest is disposed of in open lands (Oladzad et al., 2021). As a raw material, DPR contains a significant amount of dietary fiber, carotenoids, sterols, minerals, phenolic compounds such as lignans, flavonoids, phenolic acids, and quinones (Cheng et al., 2020; Struck & Rohm, 2020).

The valorization of date fruit byproducts can remarkably contribute to sustainable agro-food waste usage and help mitigate environmental pollution (Chandrasekaran, 2013). Therefore, assessing the health-promoting attributes of agro-food byproducts is a critical initial step in transforming biowaste into marketable products. Recently, in vitro digestion has been used to obtain a more precise evaluation of the health-promoting benefits of fruit/vegetable byproduct, including ACE inhibition, antioxidant and antidiabetic activities, and antiproliferative effects (Gouw et al., 2017; Ribeiro et al., 2021). Additionally, such bioaccessible portions have recently been analyzed using untargeted/targeted metabolomics via the UPLC-QTOF technique to explore metabolic pathways and identify metabolites that possess potential biological activities (Coelho et al., 2021; Stafussa et al., 2021). Analogously, an assessment of the date fruit byproduct is necessary.

Fermentation technology is a common and sustainable approach for developing high-value-added food products and preserving food (Chandrasekaran & Bahkali, 2013). The fermentation process relies on two key factors: the microorganisms employed and the substrate involved (Cuvas-Limon et al., 2020). Microorganisms play a crucial role in initiating the fermentation process of food, which involves the conversion of primary metabolites into new secondary metabolites (Cuvas-Limon et al., 2020). This transformation brings about organoleptic changes in the substrate and extends its shelf life (Tamang et al., 2016). Depending on the substrate being utilized, these

microorganisms can be bacteria, fungi, or yeasts (Bourdichon et al., 2012). The fermentation mechanism varies based on the specific substrate, which can include dairy, meat, vegetables, fish, cereals, fruits, and even food waste (Marco et al., 2017). Primary and secondary metabolites are produced during fermentation, such as antimicrobial peptides, vitamins, antibiotics, folates, carbon dioxide, organic acids, and alcohol. These compounds are referred to as bioactive compounds due to their therapeutic potential. Siriwardhana et al. (2013) reported that bioactive compounds have the ability to promote health benefits by reducing excessive oxidative stress and alleviating inflammation and metabolic disorders. In this context, microbial bioconversion can be performed using the dietary fiber and yeast-fermentable sugars present in DPR as a nutrient and carbon source for the targeted value-added products (Munekata et al., 2021; Xiang et al., 2019).

Consequently, the current work was implemented in the nonfermented and fermented DPR with the aim of evaluating the chemical characteristics, including volatile compounds, organic acids, and sugars, and quantifying phenolic compounds; assessing the bioactivities of fermented DPR (angiotensin-converting enzyme (ACE) inhibition, proteolytic activity, total phenolic compound (TPC), and activities of antidiabetic, antioxidant, and cytotoxicity) in the in vitro pre- and post-digestion (bioaccessible portions); and analyzing untargeted and carbohydrates metabolites by UPLC-QTOF.

4.3 Materials and Methods

4.3.1 Yeast Propagation, Proliferation, pH, and Titratable Acidity

Saccharomyces cerevisiae and *Pichia kudriavzevii*, with accession numbers OK441070 and OK441073, respectively, were previously isolated from traditional fermented food sources and identified as potential probiotic yeasts (Alkalbani et al., 2022). Both yeasts were subcultured in yeast extract–peptone–dextrose (YPD) broth and stored in glycerol stocks (50% *v*/*v*) at −80°C. YPD broth (Himedia Laboratories Pvt ltd, Nashik, India) was used to culture *S. cerevisiae* and *P. kudriavzevii*, separately, before being used for fermentation. The yeast population in fermented DPR and unfermented DPR (control) was counted by serially diluting with 0.1% (*w*/*v*) peptone water and using the pour-plate technique. Triplicate plates were incubated aerobically at 25°C for 6 days,

and yeast populations were counted on YPD agar at 0, 3, and 6 days. The pH value of fermented DPR and control was measured using a calibrated digital Start-3100 pH meter (OHAUS Corporation, Parsippany, NJ, USA). Additionally, samples were titrated with 0.01 N NaOH to determine the titratable acidity (TA%), which was expressed as a percentage of lactic acid.

4.3.2 Fermentation of Date Pulp Residues

DPR was generated as a side stream of a date pulp residues produced through the combination of different date cultivars. DPR was sourced from the Emirates Dates Factory, a subsidiary of Al-Foah Company located in Al Ain, United Arab Emirates. To prepare for inoculated fermentation, DPR was mixed with distilled water at a 1:10 ratio. Subsequently, the diluted DPR was inoculated with a high concentration of *S. cerevisiae* and *P. kudriavzevii*, specifically 8.0 log CFU/mL (Appendix C1). The mixture was then subjected to incubation at a temperature of 25°C for a duration of 6 days until the pH level approached approximately 4.0. Throughout the fermentation process, DPR that remained uninoculated served as a control.

4.3.3 Chemical Properties

The chemical characteristics of fermented DPR and nonfermented DPR (control) were evaluated after six days of fermentation.

4.3.3.1 Sugars and Organic Acids

A chromatographic analysis of sugars was performed as detailed by Silva et al. (2021), with minor modifications. Briefly, the analysis was carried out using SupelcoGel Ca+ column (300 × 7.8 mm; Supelco, Sigma-Aldrich, St Louis, MO, USA), at 85°C, in isocratic mode using deionized water as the mobile phase at 0.5 mL min^{-1}. Detection was performed using PDA at 195 nm.

Organic acids were analyzed using high-performance liquid chromatography (HPLC) (Chidi et al., 2018) and a gradient solvent delivery system (Thermo Scientific Dionex Ultimate 3000 Rapid Separation LC system with Chromeleon (c) Dionex version 7.2.4.8179 software, Bremen, Germany). The column used was a Supelcogel H 30 cm × 7.8 mm Cat # 59304-U at 25°C. The mobile phase was 85% (*v*/*v*) phosphoric acid (0.04

M H_3PO_4), and the flow rate was 0.4 mL/min. The PDA 3000 Dionex Photo Diode Array Detector (190–800 nm) was used for detection. The sample solution was transferred into an HPLC autosampler vial, and 20 µL of the sample extract was injected in duplicate. The concentration of organic acids in the sample was calculated from the calibration curve using external standards.

4.3.3.2 Volatile Compounds

Volatile compounds were analyzed using a procedure described by Liu et al. (2016), via a headspace (HS) solid-phase microextraction (SPME) method, combined with carboxen/poly (dimethylsiloxane) fiber (85 µm coating, Supelco, Sigma-Aldrich, Barcelona, Spain), a gas chromatography (GC)–mass spectrometer (MS), and a flame ionization detector (FID). Filtered DPR samples (1 mL) were mixed with a saturated sodium chloride solution (1 mL), and 2 mL of the mixed solution was extracted with HS-SPME at 60°C for 50 min under a rotational speed of 250 rpm/min, in a 20 mL headspace vial. Volatile compounds were separated by a capillary column (60 m × 0.25 mm I.D., Agilent DB-FFAP, Santa Clara, CA, USA) coated with a 25 mm film thickness of polyethylene glycol modified with nitroterephthalic acid. Helium was used as the carrier gas, with a flow rate of 1.2 mL/min, and the temperature program increased from 50°C (5 min) to 230°C (30 min) at a rate of 5°C/min. Volatiles were identified by comparing their mass spectra (MS) with NIST MS library, with a matching factor over 80.

4.3.3.3 Quantification of Phenolic Compounds

The quantification of phenolic compounds of fermented DPR and control samples was measured by using a Shimadzu U-HPLC system equipped with a reversed-phase analytical column of 2.1 × 50 mm, 1.7 µm particle size (Waters Acquity UPLC BEH C18, Waters Corporation, Milford, Connecticut, USA). The column oven temperature was maintained at 45°C and the flow rate was set at 0.4 µL /min throughout the experiment. Water and acetonitrile, each containing 5% formic acid, were used as mobile phases A and B, respectively. The injection volume was 2 µL, with a run time of 20 min. The gradient profile began at 5% and increased to 20% B for 6 min, then increased to 70% B for 15 min, followed by an increase to 100% B for 1 min before returning to the

starting condition from 18–20 min. The effluents were measured at multiple wavelengths (260, 270, 280, 320, 340, and 370 nm). Phenolic compounds were identified by comparing their retention times and UV–Vis spectra with phenolic standards.

4.3.4 In Vitro Digestion by INFOGEST2.0 and Bioaccessible Portion

In vitro digestion was performed on fermented DPR and nonfermented DPR using the method described by Brodkorb et al. (2019), and the bioaccessible portion was obtained by following the method of Ayyash et al. (2021a).

4.3.5 Bioactivities of Fermented DPR

The Bioactivities of Fermented DPR and nonfermented DPR samples were assessed before and after a six-day fermentation period, considering both their undigested and in vitro-digested states (bioaccessible portions).

4.3.5.1 Inhibition of α-Amylase and α-Glucosidase

The α-amylase and α-glucosidase inhibition assays of the samples were carried out according to the method described by Ng et al. (2020) for α-amylase and Ho et al. (2022) for α-glucosidase. The following equation was used to calculate the inhibition percentage:

$$\text{Inhibition } \% = \left(1 - \frac{Abssample - Absblank}{Abscontrol}\right) \times 100$$

4.3.5.2 Antioxidant Capacity

The DPPH (1,1-diphenyl-2-picrylhydrazyl) and ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) ($ABTS^{\bullet+}$)) radical scavenging assays were conducted following the method outlined by Ng & Kuppusamy (2019). The percentage of radical scavenging activity was determined using the following calculation:

$$\text{Scavenging rate } \% = \left(\frac{Absblank - Abssample}{Absblank}\right) \times 100$$

4.3.5.3 Cytotoxicity Activities

The samples of the bioaccessible portion were assayed against Caco-2 and MCF-7 carcinoma cell lines according to the method detailed by Ayyash et al. (2021a). The cytotoxicity percentage was determined using the following equation:

$$\text{Antiproferative activity (\%)} = \left[1 - \frac{Rsample - R0}{Rctrl - R0}\right] \times 100$$

4.3.5.4 Free amino acid contents and TPC

The *O*-phthaldialdehyde (OPA) assay was conducted to estimate the total free amino acids as described in the method of Ayyash et al. (2021a). The TPC of the samples was measured using the Folin–Ciocalteu reagent method (Kashyap et al., 2022).

4.3.5.5 ACE Inhibition

ACE inhibition activity was assessed according to the method described by Liu et al. (2018a). The ACE inhibition activity (%) was calculated as:

$$\text{ACE inhibition \%} = \left(1 - \frac{Ab - Aa}{Ab - Ac}\right) \times 100$$

Ab refers to the absorbance without adding the sample solution (buffer solution added instead of the sample), and *Aa* refers to the absorbance in the presence of ACE and the sample solution. *Ac* refers to the absorbance of the blank (HCl was added before the addition of ACE).

4.3.6 Untargeted Metabolomics and Carbohydrate Metabolomics Analyses

4.3.6.1 Untargeted Metabolite Analysis

The UPLC-QTOF technique was employed to analyze the bioaccessible fractions of fermented DPR by *P. kudriavzevii* and control on the sixth day. The analytical system employed for separation and identification of metabolites consisted of a Waters UPLC I-Class Plus (Waters, USA) coupled with a Q Exactive high-resolution mass spectrometer (Thermo Fisher Scientific, USA). A Waters ACQUITY UPLC BEH C18 column (1.7 µm, 2.1 mm × 100 mm, Waters, USA) maintained at a temperature of 45°C was utilized for chromatographic separation. The mobile phase composition varied depending on the

ionization mode: 0.1% formic acid (A) and acetonitrile (B) were used in positive mode, while 10 mM ammonium formate (A) and acetonitrile (B) were used in negative mode. For mass spectrometry, Q Exactive (Thermo Fisher Scientific, USA) was used for primary and secondary data acquisition. The equipment had a scanning range from 70 to 1050 *m*/*z* with a resolution of 70,000. During the MS acquisitions, the AGC target was set to 3×10^6, and the resulting data were analyzed using Compound Discoverer 3.3 (Thermo Fisher Scientific, USA) in combination with bmdb (BGI metabolome database), mzcloud database, and chemspider online database. The output data matrix contained details such as metabolite peak area and identification results, which were further subjected to analysis and processing. The software utilized for this purpose was Compound Discoverer, version v.3.3, with the following parameters: parent ion mass deviation: <5 ppm, mass deviation of fragment ions: <10 ppm, and retention time deviation: <0.2 min. Additional information can be found on the official website: https://mycompounddiscoverer.com (21 March 2023).

4.3.6.2 Carbohydrate Metabolites Analysis

To extract metabolites, 25 mg of bioaccessible samples were weighed and subjected to extraction using a precooled extraction reagent composed of methanol, acetonitrile, and water (2:2:1, *v*/*v*/*v*), with the addition of internal standards mix 1 (IS1) and internal standards mix 2 (IS2) to ensure sample preparation quality control.

The samples were homogenized for 10 min, sonicated, and incubated at -20°C for 1 h before being centrifuged and the supernatant was then transferred for vacuum freeze drying. The metabolites were resuspended in 10% methanol and analyzed using LC-MS/MS with a 2D UPLC (waters, USA) and a tandem Q-Exactive mass spectrometer (Thermo Fisher Scientific, USA) with a heated electrospray ionization (HESI) source. The analysis was controlled by the Xcalibur 2.3 software program (Thermo Fisher Scientific, Waltham, MA, USA). The chromatographic separation and mass spectrometry were carried out as described in Section 2.6.1.

4.3.7 Data Processing

To perform a comprehensive analysis of the mass spectrometry data, the commercial software Progenesis QI (Waters, Manchester, UK) and an in-house metabolomics analysis process were utilized. Metabolite identification was conducted by referencing the HMDB (http://www.hmdb.ca/ (21 March 2023)) and KEGG (https://www.genome.jp/kegg/ (21 March 2023)) databases. Principal component analysis (PCA) and heatmap analysis were employed for further data exploration. For metabolite identification, the BMDB Library (BGI Metabolome Database), an internally developed database, was utilized. This database encompasses retention time (RT), MS1 spectrum (MS1), and MS2 spectrum (MS2) information for all standards. It covers key primary metabolites and metabolic intermediates involved in crucial metabolic pathways, such as carboxylic acids, amino acids, biogenic amines, polyamines, nucleotides, coenzymes and vitamins, monosaccharides and disaccharides, fatty acids, lipids, steroids, and hormones.

Furthermore, the HMDB database, known for its extensive coverage of human metabolites, was also employed in the analysis. It provides datasets across three categories: chemical data, clinical data, and analytical biology and biochemical data. Both water-soluble and fat-soluble metabolites are included in this resource. Additionally, the provided information includes links to other databases such as KEGG and PubChem, facilitating convenient access to further relevant information.

4.3.8 Statistical Analysis

All results were expressed as the mean of three independent determinations ± standard deviation (SD), unless otherwise mentioned. The statistical mean difference among the samples was determined by analysis of variance (ANOVA) with Tukey post-test [30]. PCA for variables and observations was conducted to estimate the structural correlation of the variables and to indicate the relationship between the fermented DPR and control. The statistical analyses were performed by XLSTAT software (Addinsoft, New York, NY, USA).

4.4 Result and Discussion

4.4.1 Yeast Proliferation, pH, and Titratable Acidity

The two yeast strains (*S. cerevisiae* and *P. kudriavzevii*) maintained high proliferation, more than 7.0 log CFU/mL, throughout 6 days of fermentation (Appendix C.1-A). In contrast, yeast growth in the nonfermented DPR (control) remained low, at around one log CFU/mL over 6 days, indicating that the two yeast strains were suitable for the DPR environment. Appendix C.1-B shows that the pH of the fermented DPR decreased from an average of 5.26 to below 4.1 over 6 days ($p < 0.05$), while the pH of the nonfermented DPR remained relatively unchanged at an average of 5.4 during the six days. The decreasing pH in the sample fermented by *P. kudriazevii* was slightly faster than that in *S. cerevisiae* ($p > 0.05$). In line with the pH value, an increase in titratable acidity (Appendix C.1-C) was observed in the fermented samples during the fermentation period ($p > 0.05$). The alterations in pH and titratable acidity could be explained by the increased amounts of organic acids synthesized during yeast fermentation.

4.4.2 Chemical Properties

4.4.2.1 Evaluation of Sugars and Organic Acids

Chromatographic analysis of sugars in the fermented DPR showed that sucrose, glucose, fructose, and arabinose were not present in significant amounts, likely due to their consumption by *S. cerevisiae* and *P. kudriavzevii* during fermentation. Glucose is metabolized into organic acids via the glyoxylate and tricarboxylic acid (TCA) cycles (Chidi et al., 2018).

In this study, six organic acids were studied (Figure 13) in nonfermented DPR (control) and fermented DPR by two yeast strains after 6 days of fermentation. These organic acids included oxalic acid, citric acid, malic acid, lactic acid, acetic acid, and propionic acid. Compared to the control, all screened organic acids showed an increase in concentration, except for oxalic acid which decreased in two samples of fermented DPR. Additionally, propionic acid was newly produced in fermented DPR by *S. cerevisiae*. The levels of lactic acid and malic acid in fermented DPR by *S. cerevisiae*

(748.3, 4359.1 mg/100 g) and *P. kudriavzevii* (1698.6, 2463.5 mg/100 g) were significantly higher than their corresponding controls (475, 234.5 mg/100 g), respectively, indicating that yeast fermentation significantly promoted the synthesis of lactic and malic acids. The sample fermented by *P. kudriavzevii* had slightly higher amounts of citric and acetic acids (244.8, 185.5 mg/100 g) than the sample fermented by *S. cerevisiae* (195.8, 154.8 mg/100 g), respectively.

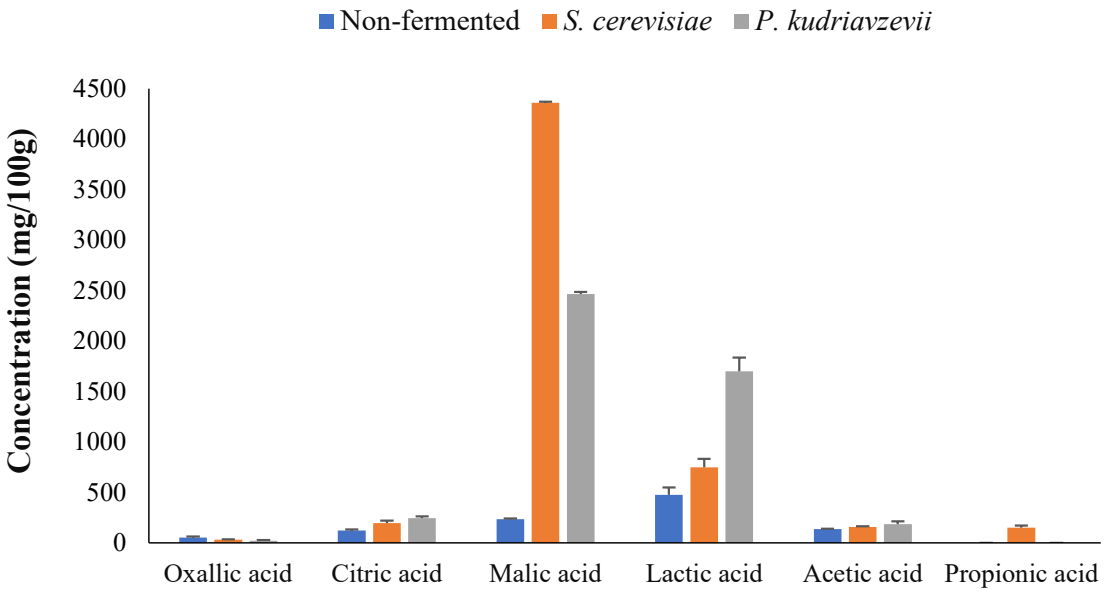

Figure 13: Concentrations of organic acids (mg/100 g) in nonfermented (control) and fermented date pulp residues by *S. cerevisiae* and *P. kudriavzevii*.
Values are the mean values (n = 3). Error bars express standard deviations.

Differences in organic acid content are influential factors that contribute to variations in flavor among different varieties of date fruits (Flowers et al., 2022). In this aspect, Ghnimi et al. (Ghnimi et al., 2018) and Elshibli and Korpelainen (Elshibli & Korpelainen, 2009) described that each variety of date fruit has its own pattern of organic acid content. However, malic acid has been identified as a common acid with varying levels in date fruits (Farag et al., 2014; Kamal-Eldin & Ghnimi, 2018).

In the present work, the high concentration of lactic acid and acetic acid in fermented DPR compared to the control was related to the sugar-metabolizing pathway of two yeast strains (*S. cerevisiae* and *P. kudriavzevii*). These yeast strains enabled the conversion of sugars in DPR into lactic acid, ethanol, acetic acid, and CO_2. The increase

in malic acid and citric acid may have resulted from the accumulation of these acids as byproducts of yeast fermentation through the oxidative pathway of the tricarboxylic acid (TCA) cycle (Wei et al., 2021; Zelle et al., 2008). As shown in Figure 13, malic acid was the predominant organic acid in the fermented samples, associated with the tart, mellow, smooth, and sour taste, as well as giving the date its distinguished fruity taste (Al-Farsi et al., 2005; Farag et al., 2014). In line with our results, Alahyane et al. (Alahyane et al., 2022) and Cherif et al. (Cherif et al., 2021) reported an abundance of malic acid in date palm fruits.

4.4.2.2 Evaluation of Volatile Compounds

Although the influence of yeast fermentation on the volatile profiles has been extensively studied in different food products, especially wine (Canonico et al., 2019; Lee et al., 2017; Torrens et al., 2008), there is still a need to understand the effect of selective yeast fermentation on the volatiles of date pulp residues (DPR). Figure 14 shows the chemical categories of volatile compounds that were detected in both fermented and nonfermented DPR. In total, 42 volatile compounds were identified, including 10 volatile acids, 8 esters, 6 alcohols, 4 phenols, 3 ketones, 4 aldehydes, and 10 compounds belonging to various other chemical groups (Appendix C.2). Figure 14 highlights that when *S. cerevisiae* and *P. kudriavzevii* were used to ferment DPR, volatile compounds belonging to the alcohol group were synthesized in higher amounts (~41% and 28% RPA, respectively) compared to the control (~11% RPA). On the other hand, the RPA% of esters decreased by 18% in the samples fermented with *S. cerevisiae* compared to the control (~30.4%).

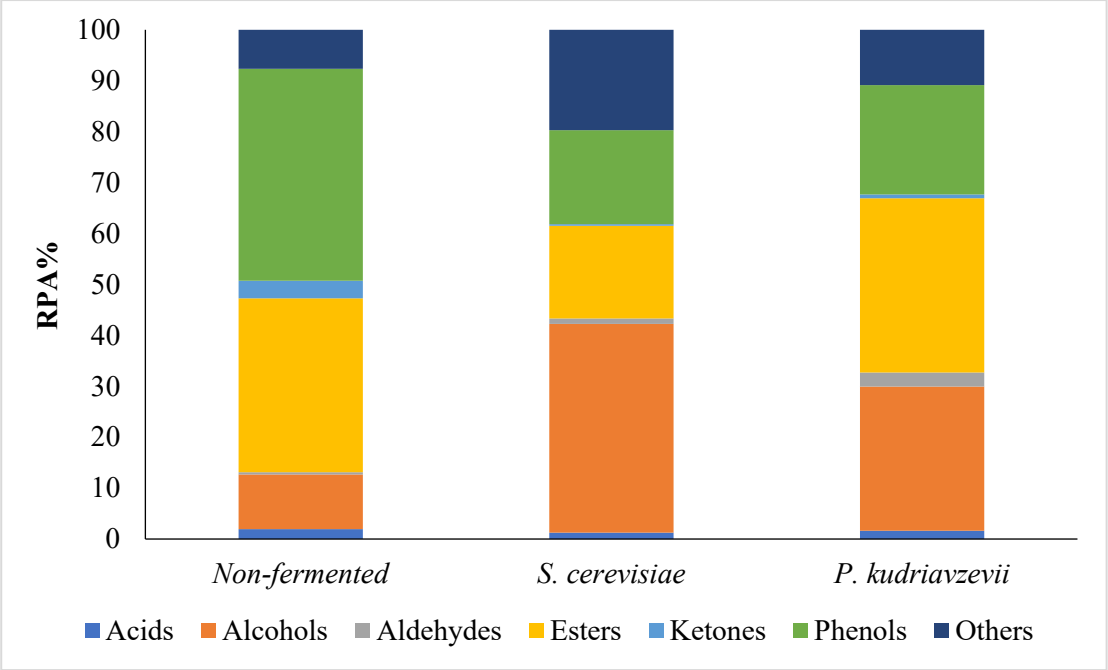

Figure 14: Chemical classes of volatile compounds identified in nonfermented and fermented date pulp residues, represented in % relative peak area (RPA).

Among the volatile acids, acetic acid had a comparably higher level, with greater amounts found in the *P. kudriavzevii* sample, which is compatible with the results of our organic acids analysis (Figure 13).

Varied patterns were noted for the formation of volatile alcohols (Appendix C.2). Isobutanol was newly synthesized in two fermented samples, with the highest level observed in the *P. kudriavzevii* sample. The production of this alcohol can be attributed to the degradation of valine in the yeast cytosol via the Ehrlich pathway (Lakshmi et al., 2021). The concentrations of 2-phenylethanol and isoamyl alcohol were significantly increased after fermentation, with the highest level found in the sample fermented by *S. cerevisiae*. The increasing levels of these alcohols (2-phenylethanol, isoamyl alcohol) could be derived from the essential amino acids phenylalanine and leucine (Abe & Horikoshi, 2005; Ravasio et al., 2014), respectively, which might be present in the fermented DPR.

An increasing trend was noted in the class of esters after fermentation (Appendix C.2). Among all esters, ethyl acetate was the prevalent volatile ester in all samples, which may impart a solvent-like aroma if in excess. It has been documented that the

yeast generation of volatile acetates, such as isoamyl, ethyl, and 2-phenethyl acetate, occurs through alcohol acetyltransferases that use acetyl-CoA to acetylate various alcohols (Shalit et al., 2001; Yahyaoui et al., 2002). Verstrepen et al. (Verstrepen et al., 2003) and Saerens et al. (Saerens et al., 2008) reported that the formation of esters in fermented foods by yeast depends on the yeast strain used, which may clarify the variations in ester production between the two yeast strains utilized in the present study (*S. cerevisiae* and *P. kudriavzevii*). Among all the volatile phenols, 2,4-di-*tert*-butylphenol was the most abundant in the fermented samples.

Previous studies, such as those conducted by Siddeeg et al. (Siddeeg et al., 2019), Saafi et al. (Saafi et al., 2022), and Flowers et al. (Flowers et al., 2022), have described the volatile compounds present in date palm fruits. However, what sets our work apart is its focus on the evaluation of the volatile compounds generated in date pulp residues after yeast fermentation. This key difference may explain some of the discrepancies between our findings and those of previous studies.

4.4.2.3 Phenolic Compounds Profile

Phenolics derived from food have been linked to several health benefits (Rashmi & Negi, 2020). Fermentation has the ability to change the phenolic composition of plant-based foods by releasing specific phenolic monomers from their complex structures (Leonard et al., 2021). Yeast enzymes, such as pectinases and β-glucosidase (β-glucoside glucohydrolase, EC 3.2.1.21), play a role in facilitating the release of these phenolic monomers (Zhang et al., 2021).

In our work, a total of ~70, 194, and 204 mg/kg of 20 phenols were quantified by the UPLC in nonfermented DPR (control), and fermented DPR by *P. kudriavzevii* and *S. cerevisiae*, respectively (Figure 15). In general, yeast fermentation significantly increased the level of phenolic compounds from the byproduct, especially gallic acid, tyrosol, syringic acid, and (-)-epicatechin. Following fermentation, two new flavonoids were detected, namely, apigenin in small quantities and (-)-epicatechin in dominant quantities. UPLC analysis suggests that these two flavonoids might have been synthesized by the yeast strains used in fermenting the date pulp residues.

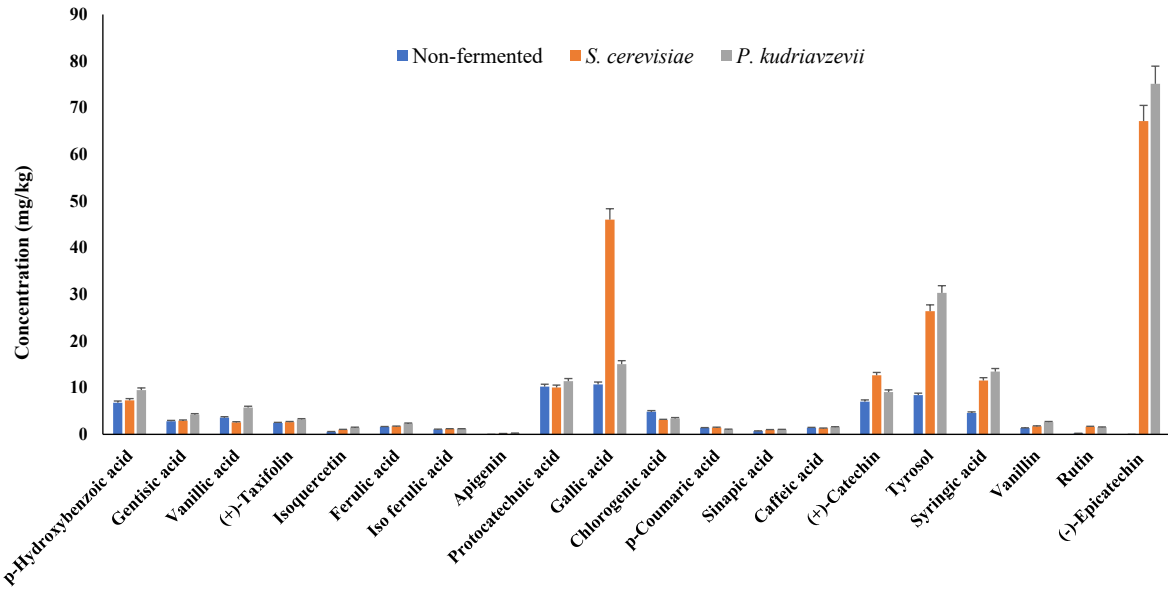

Figure 15: Concentrations of phenolic compounds (mg/kg) of nonfermented and fermented date pulp residues measured by U-HPLC.
Values are the mean values (n = 3). Error bars express standard deviations.

In the control sample, the proportions of flavonoids, phenolic acids, and other phenols (phenylethanoid and phenolic aldehyde) were approximately 15%, 71%, and 14% of total phenols, respectively. In the sample fermented by *P. kudriavzevii*, the proportion changed to ~47%, 36%, and 17%, while in the sample fermented by *S. cerevisiae*, it was ~42%, 44%, and 14%, respectively, of total phenols. It is evident that fermentation significantly improved the contribution of flavonoids to the total phenols at the expense of the proportion of phenolic acids. (-)-Epicatechin, a flavan-3-ol compound, had the highest concentration among all phenols in the fermented DPR samples, with levels of 75 and 67 mg/kg, respectively, whereas it was not detected in the control sample. This increase in the level of (-)-epicatechin by fermentation may be attributed to its release from oligomeric and polymeric proanthocyanidins and melanin (Alam et al., 2022; Hammouda et al., 2013). Interestingly, the production of gallic acid, a hydroxybenzoic acid derivative, significantly increased from 10.7 mg/kg to 46.0 mg/kg in the sample fermented by *S. cerevisiae*, and was enhanced to 15.0 mg/kg in the sample fermented by *P. kudriavzevii*. The phenylethanoid compound, tyrosol, underwent considerable development after fermentation, increasing from 8.4 mg/kg to 30.3 and 26.4 mg/kg in samples fermented by *P. kudriavzevii* and *S. cerevisiae*, respectively. Tassoult et al. (Tassoult et al., 2021) determined the presence of tyrosol in various Algerian date

fruit varieties and date pastes (ranging from 8.17–16.37 mg/kg and 16.12–16.57 mg/kg, respectively); however, the quantity of tyrosol in our study was notably higher. This disparity may be mainly ascribed to yeast metabolites secreted during fermentation, as several studies have described the production of tyrosol, during alcoholic fermentation by different yeast species (Lasanta et al., 2021; Liu et al., 2021) via the Ehrlich pathway.

Consistent with our results, previous studies reported by Alshwyeh (Alshwyeh, 2020), Amira et al. (Amira et al., 2012), and Zihad et al. (Zihad et al., 2021) have identified most of these phenols in date palm fruits in varying quantities. However, differences between our findings and others in terms of the quantity and type of phenols, organic acids, and volatile compounds may be attributed to the effects of yeast metabolism, variations among date varieties, and date product matrices (date fruits and date pulp residues), as well as external factors such as geographic origin, environmental conditions (e.g., sunlight, fertilizers), and extraction methods.

4.4.3 Bioactivities of Fermented DPR

Selective fermentation is one of the proposed solutions for reducing food waste, achieving sustainable food production, and creating novel food products, all within an integrated circular economy. In this regard, the current paper evaluates the health benefits of fermented DPR in a bioaccessible portion. As illustrated in Appendix C.3-A, in the bioaccessible portions, the TPC for all samples (*S. cerevisiae*, *P. kudriavzevii*, control) averaged 9.3 mg GAE/mL. Appendix C.3-A describes that the TPC in the bioaccessible portions had a significant ($p < 0.05$) decline compared to undigested samples and a slight decline in the fermented DPR compared to the control. In general, the DPR fermented by two yeast strains had a higher OPA than the control (Appendix C.3-B). For the *S. cerevisiae*, *P. kudriavzevii*, and control samples, the OPA absorbances measured before digestion were 0.51, 0.65, and 0.40. These absorbances decreased sharply in the bioaccessible portions to 0.34, 0.40, and 0.23, respectively.

Figure 16(A–D) exhibits the antidiabetic activities inhibitions (A), antioxidant capacities (B), ACE inhibition (C), and cytotoxicity capability (D) of nonfermented and fermented DPR for undigested samples and bioaccessible portions. Our data revealed that the bioactivities of fermented DPR showed different trends in the results of in vitro

post-digestion (bioaccessible portions) compared to predigestion. In the bioaccessible portion, α-amylase inhibition and cytotoxicity activities showed improved levels. However, antioxidant capacities, TPC, and OPA exhibited a decline, whereas ACE inhibition had comparatively constant levels in the bioaccessible portion.

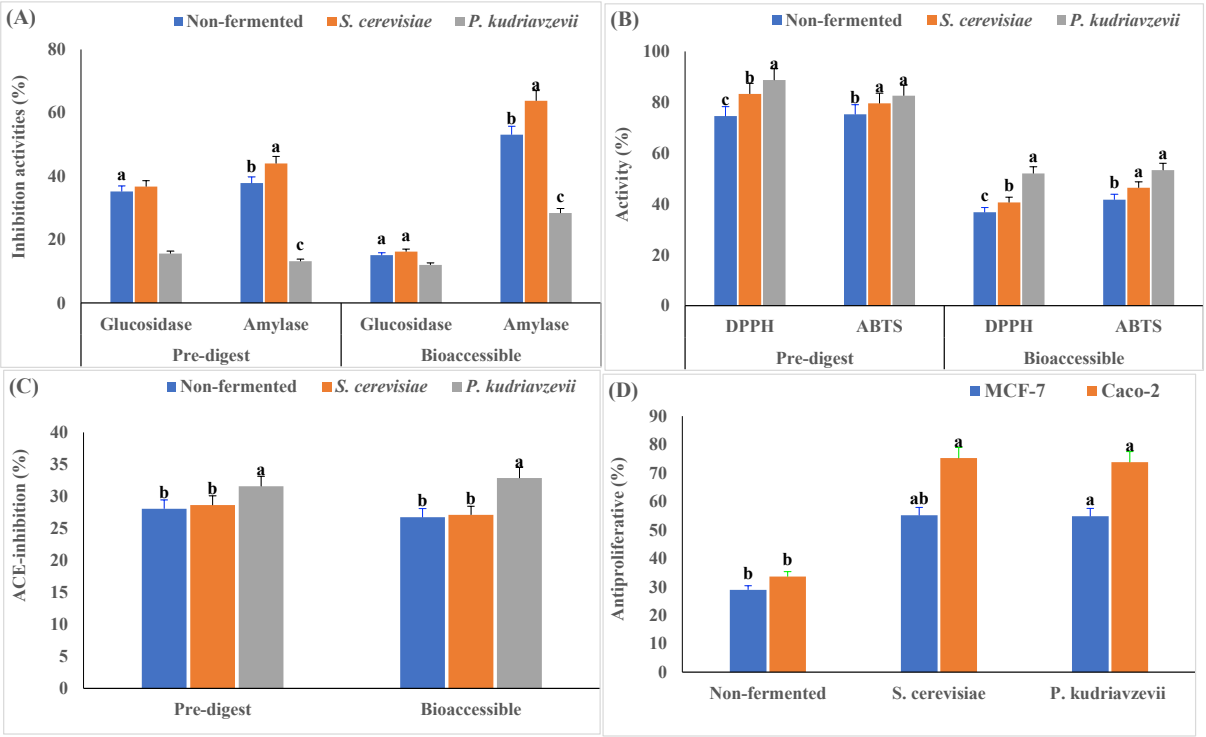

Figure 16: Assays of health-promoting benefits of nonfermented and fermented date pulp residues for undigested samples and bioaccessible portions.
Antidiabetic activities inhibitions (A), antioxidant capacities (B), ACE inhibition (C), and cytotoxicity capability (D). Values are the mean values (n = 3). Error bars express standard deviations. a-c: mean values with different lowercase letters different significantly at $p < 0.05$.

The unchanged and upward trends of ACE inhibition and α-amylase/cytotoxicity inhibition activities in the bioaccessible portions of fermented DPR propose a quantitative conservation and/or development in the bioactive compounds responsible for these features throughout the digestion process. The strain *S. cerevisiae* had a significantly higher α-amylase inhibition than *P. kudriavzevii*. The relatively high inhibitory effect of α-amylase in our study is in accordance with that found by Barros et al. (Barros et al., 2020) for a bioaccessible portion of fermented soursop residue by multiple bacterial probiotics.

In contrast, downward trends in the examined nutraceutical properties (OPA, TPC, α-glucosidase inhibition, and antioxidant activities) were observed at various levels in the bioaccessible portion. These reductions could be mainly attributed to exposure to digestive conditions with a changed pH and digestive enzymes, resulting in the degradation of bioactive compounds (Ketnawa et al., 2022). Moreover, during digestion, phenolic compounds are generally sensitive to interactions with specific dietary constituents such as minerals, dietary fiber, and macromolecules (Jakobek, 2015; Sęczyk et al., 2021). Thus, the physicochemical and structural properties of nutrients can be altered through these interactions, affecting their bioaccessibility (Ozdal et al., 2013). In this context, the antioxidant capacities may decrease in the bioaccessible portion due to the conversion of antioxidant phenolic compounds into different compounds with new chemical properties (Seraglio et al., 2017). Another potential explanation is that during digestion, the polyphenols that are linked to health-promoting benefits usually bind to the food matrix (Shahidi & Naczk, 2003). Consequently, the high amount of insoluble phenolic compounds present in the bound fiber of date pulp residues might not be fully digested, as was observed in this trial.

Likewise, in line with our results, Andrade Barreto et al. (Andrade Barreto et al., 2023) reported that the antioxidant capacities of fermented orange juice by *S. cerevisiae* were preserved in reasonable percentages in the bioaccessible portion. On the contrary, the results obtained by Djaoudene, Mansinhos (FAO/WHO, 2002) showed a significant enhancement in TPC and antioxidant capacities for Algerian date extracts after being subjected to in vitro digestion. However, the differences between our findings and others could be ascribed to the differences in the metabolic activities during microbial fermentation (Sharma et al., 2020), which definitely varies according to the microorganism used. In addition, the specificity of the screened food matrix plays a role in obtaining different results in terms of its content of bioactive compounds and the structural alterations that take place on those compounds during digestion (Sęczyk et al., 2021).

4.4.4 Untargeted and Carbohydrates Metabolomics Analyses

4.4.4.1 Untargeted Metabolite Analysis

The untargeted metabolomics methodology permits the investigation and classification of a wide spectrum of metabolites in a sample, irrespective of prior knowledge of their identities. This approach offers valuable knowledge concerning the chemical composition of fermented and unfermented DPR and their possible impacts on human health.

Due to the relatively superior antioxidant capacities of the *P. kudriavzevii* sample compared to the *S. cerevisiae* sample, it was selected, along with the nonfermented DPR (control), to conduct this part of the study. Figures 17(A) and 17(B) exhibit the outcomes of PCA and heatmap analyses, respectively, of metabolites differentially present in fermented DPR by *P. kudriavzevii* and the control in the bioaccessible portions of the sixth day of fermentation. The PCA analysis (Figure 17(A)) displays the observed dissimilarities between the nonfermented DPR (control) and *P. kudriavzevii* samples, indicating the differences in metabolites between these two groups. PC1 and PC2 represented 91.78% and 5.38% of the total variations, respectively. Furthermore, the unsupervised hierarchical clustering method shows that the three replicates of each experimental treatment formed a separate cluster, with the *P. kudriavzevii* sample forming a cluster distinct from the control sample (Figure 17(B)). Figure 17(B) illustrates the bioaccessible metabolites grouped into two significant clusters, G1 and G2, reflecting the differences in metabolite concentrations between these groups. Notably, metabolites in G1 and G2 showed a significant variation in *P. kudriavzevii* sample compared to the control. Additionally, the volcano plot (Appendix C.4) depicts the differential metabolites classified as upregulated, downregulated, and nonsignificant. According to Appendix C.4, in the fermented DPR by *P. kudriavzevii*, 165 metabolites were upregulated, 244 were downregulated, and 918 showed no significant differences compared to the control.

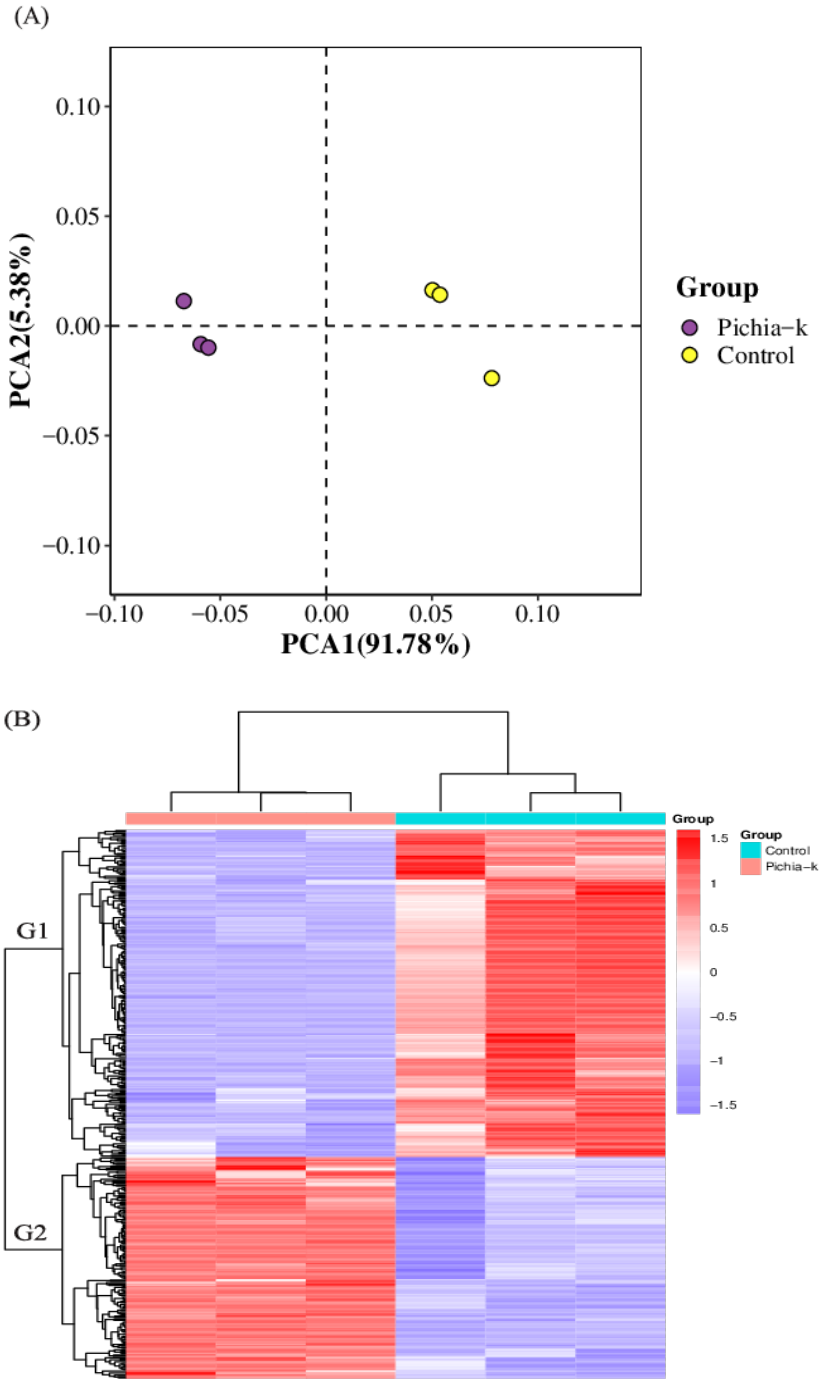

Figure 17: PCA scores (A) compare *P. kudriavzevii* with control, and heatmap (B) illustrates the metabolite concentration.

The colors on the heatmap range from violet to red, and their specific color references are explained in the online version of the article. The cluster analysis in panel (B) presents the expression patterns of the differential metabolites in the two sample groups. Each row in the heatmap denotes a differential metabolite, whereas each column indicates a sample. The color scale spans from violet to red, where violet represents a low expression level, and red reflects a high expression level.

In our study, the results of the PCA (Figure 17(A)) reveal significant differences in the metabolite profiles of the *P. kudriavzevii*-fermented sample compared to the control group. Furthermore, the heatmap in Figure 17(B) provides further support for this observation by highlighting disparities in both the types and levels of metabolites between the two samples. These results suggest that during DPR fermentation, the biological actions of *P. kudriavzevii* may have led to the synthesis of novel bioactive compounds or the enhancement of present ones. Specifically, the *P. kudriavzevii* sample showed an increase in the concentrations of metabolites in the G2 group, while those in the G1 group decreased compared to the control. Therefore, it is likely that *P. kudriavzevii* metabolized some of the compounds in the G1 group while simultaneously increasing the production of those in the G2 group during DPR fermentation.

4.4.4.2 Metabolic Pathway Analysis

By performing metabolic pathway enrichment analysis on differential metabolites using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, significant alterations in metabolic pathways can be identified, contributing to the interpretation of biological phenotypes.

In our paper, metabolic pathways with a $p < 0.05$ were regarded as highly enriched with different metabolites. To showcase the top 10 enriched metabolic pathways, the study employed bubble charts, as seen in Figure 18, which revealed the smallest *p*-values. It is noteworthy that the C5-branched dibasic acid metabolizing pathway had a significant increase in differential metabolites, with four metabolites identified in this pathway, including itaconate, L-glutamic acid, oxoglutaric acid, and (+/-)-2-hydroxyglutaric acid (Appendix C.5). Bambouskova et al. (Bambouskova et al., 2018) and Mills et al. (Mills et al., 2018) described the anti-immune and anti-inflammatory mechanisms of itaconate. L-glutamic acid, a nonessential amino acid, exhibits antioxidant activity (Zhang et al., 2022), while oxoglutaric acid has the ability to boost the production of collagen and may have an impact on processes related to aging (Zhou et al., 2021). Seven metabolites, such as L-tryptophan, indole-3-acetic acid, and skatole, were detected in the tryptophan metabolizing pathway. Generally, the results indicated that fermentation had a significant impact on the amino acids.

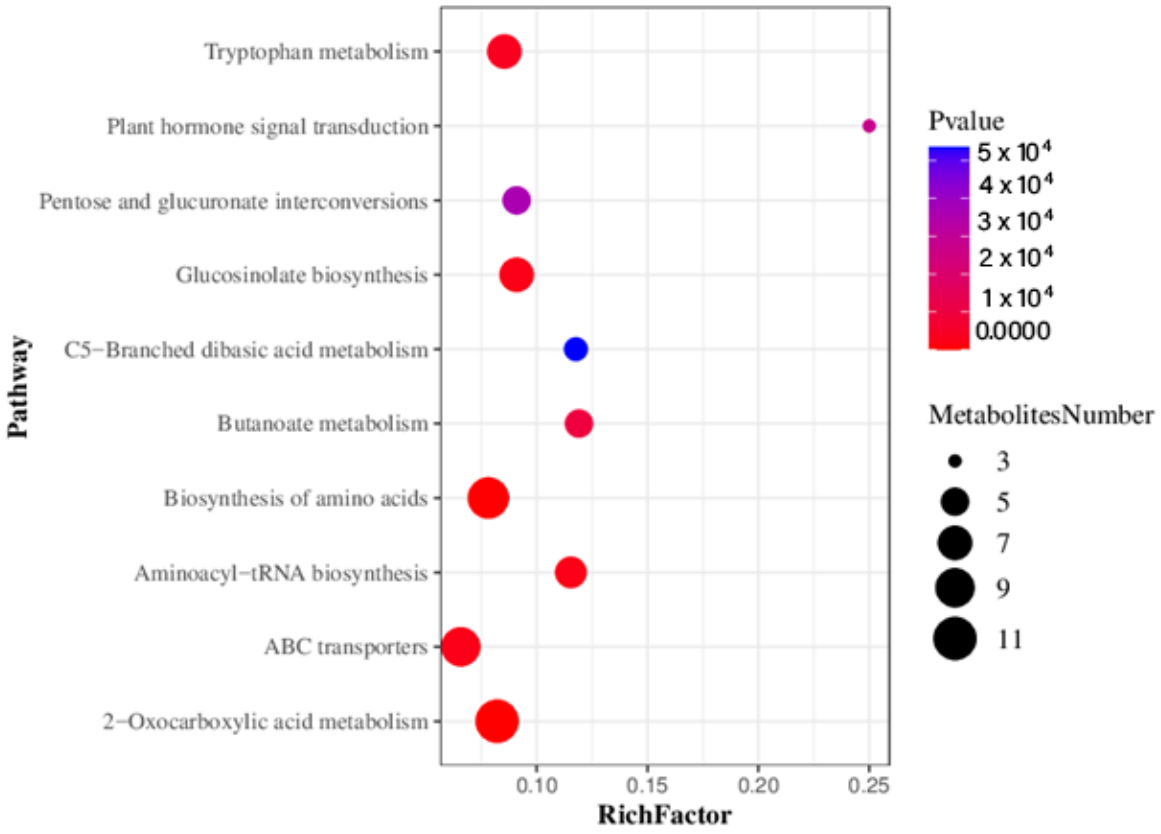

Figure 18: The x-axis shows the RichFactor, representing the proportion of differential metabolites in a pathway relative to all annotated metabolites in the same pathway.

The dot size reflects the number of differential metabolites annotated within the pathway. The y-axis represents the name of the KEGG metabolic pathway. A higher RichFactor value indicates a higher ratio of annotated differential metabolites in the pathway. The size and color of the dots reflect the number and level of annotated differential metabolites within the pathway.

4.4.4.3 Carbohydrate Metabolites Analysis

Figure 19 presents the analysis of carbohydrate metabolism (A) and the identification of pathways correlated with differentially regulated metabolites using the KEGG database (B). In Figure 19(A), the differentially regulated metabolites between the *P. kudriavzevii* sample and the control were classified into lipids, compounds with biological roles, phytochemical compounds, and others that remained uncategorized. The most significant categories of differential metabolites were lipids (8), followed by amino acids, peptides, and analogues (7), and terpenoids (3). Figure 19(B) displays the major metabolic pathways of carbohydrate identified through the KEGG database analysis, which include global and overview maps (12), amino acid metabolism (5), lipid

metabolism (5), biosynthesis of other secondary metabolites (3), and membrane transport (3).

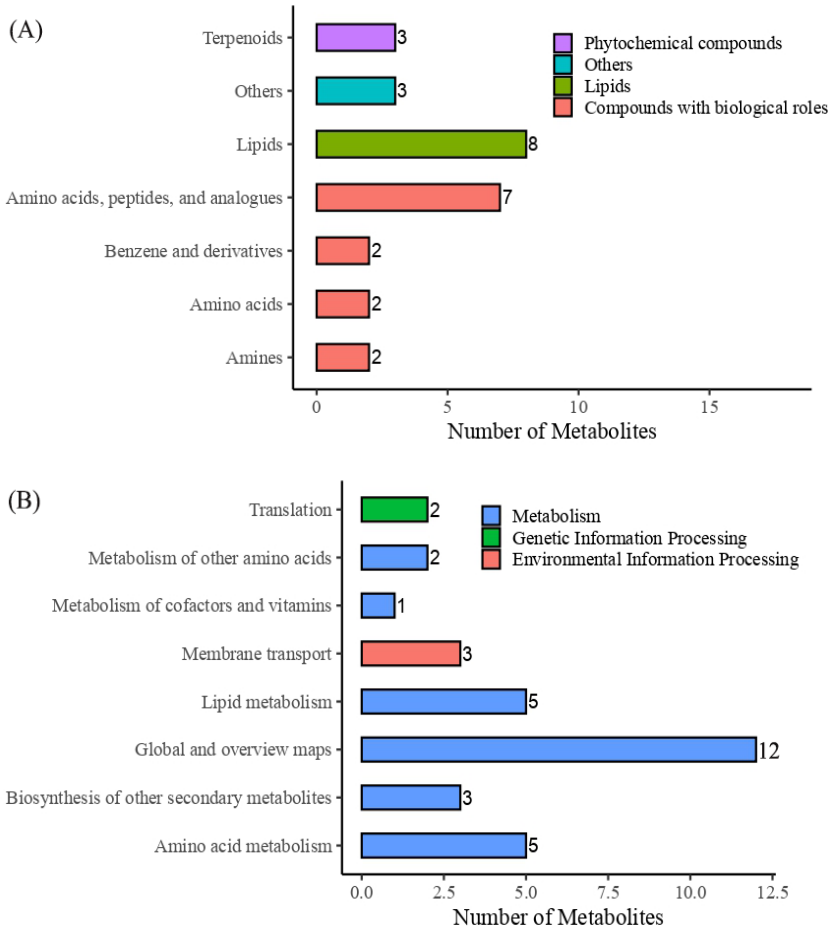

Figure 19: Identification of differential carbohydrate metabolites (A) and analysis of their KEGG pathway (B) in the bioaccessible portions of the *P. kudriavzevii* sample and the control.

The findings obtained from Figure 19(A) indicate that the bioaccessible portions contained a diverse range of compounds, including amino acids, benzene and derivatives, amines, and peptides, all of which possess biological activities. Furthermore, the LC-QTOF analysis of bioaccessible portions revealed the presence of three metabolites, including tutinolide, triterpenoids, and monoterpenoids, that belong to the terpenoid class of phytochemical compounds. Terpenoids have been extensively studied and found to have significant effects on human health (Masyita et al., 2022). For example, monoterpenoids exhibit antibacterial properties that can disrupt microbial multiplication and development, as well as interfere with their physiological and

metabolic processes (Burt, 2004). On the other hand, triterpenoids have been shown to possess a variety of biological properties such as antiulcer, anti-inflammatory, antibacterial, hepatoprotective, antiviral, antiatherosclerotic, immunomodulatory, and cholesterol-lowering effects (Akihisa et al., 2001; Szakiel et al., 2012). Overall, the majority of metabolomics findings suggested that fermentation significantly enhanced the functions of DPR.

4.5 Conclusions

Fermentation of date pulp residues (DPR) with yeast isolates enhanced its health-promoting properties by improving its chemical composition and maximizing its potential effects. The yeast strains used in the fermentation process efficiently converted the sugars in DPR, resulting in elevated levels of lactic acid and acetic acid in the fermented DPR. *S. cerevisiae* produced more alcoholic compounds, while *P. kudriavzevii* resulted in higher levels of ester compounds, highlighting the importance of selecting the appropriate fermentation agent to manipulate the chemical composition of volatile compounds in DPR. The content of phenols increased in DPR samples following fermentation, especially gallic acid, tyrosol, syringic acid, and (-)-epicatechin. This increase may have positive impacts on the potential health-promoting attributes of fermented DPR compared to nonfermented DPR. Evaluating the bioaccessibility of fermented DPR is crucial for ensuring its usability in metabolism, and assessing the health-promoting properties of the bioaccessible portion is necessary to fully understand the potential benefits of DPR. Fermentation of DPR using yeast species can enhance the health benefits of DPR, such as amylase inhibition and cytotoxicity. Moreover, the fermented DPR (undigested samples and bioaccessible portions) exhibited higher antioxidant activities compared to the control, providing more opportunities to utilize date byproducts. The findings of the untargeted metabolomics analysis suggest that fermentation has a significant impact on the C5-branched dibasic acid metabolizing pathway, while the analysis of carbohydrate metabolites revealed the presence of three compounds belonging to the terpenoid class of phytochemicals: tutinolide, triterpenoids, and monoterpenoids. These findings indicate that fermentation can enhance the functions of DPR.

Author Contributions: N.S.A., writing—original draft, investigation, data curation, formal analysis; T.M.O., A.O., A.A.-N., S.-Q.L., and R.S.O., writing—review and editing; M.Z.A., investigation; A.K.E., investigation, writing—review and editing, M.A., conceptualization, funding, supervising, writing—review and editing, supervision. All authors have read and agreed to the published version of the manuscript.

Funding: This research and APC were funded by the United Arab Emirates University (UAEU), Al-Ain, United Arab Emirates.

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

Chapter 5: Unraveling the Potential Nutritional Benefits of Fermented Date Syrup By-product: Untargeted Metabolomics and Carbohydrate Metabolites of *In Vitro* Digested Fraction

Redrafted from

Alkalbani, N.S.; Alam, M.Z.; Al-Nabulsi, A.; Osaili, T.M.; Obaid, R.R.; Liu, S.-Q.; Kamal-Eldin, A., & Ayyash, M. Unraveling the Potential Nutritional Benefits of Fermented Date Syrup Waste: Untargeted Metabolomics and Carbohydrate Metabolites of In Vitro Digested Fraction. Food Chemistry, (Manuscript Submitted and Accepted).

5.1 Abstract

Valorization of fruit by-products is an important area of research for innovative bio-based products. This study examined the chemical properties and health-promoting benefits of fermented by *Pichia cecembensis* or *Issatchenkia orientalis*, and unfermented date syrup waste (FDSW and CDSW) and identified their metabolomics profiles after in vitro digestion. FDSW had 42 volatile compounds, including 9 new ones. FDSW had (-)-epicatechin, tyrosol, and gallic acid. Bioaccessible portions of fermented samples showed 40.7% and 53.9% α-amylase inhibition, respectively, surpassing CDSW. FDSW demonstrated superiority in cytotoxicity against Caco2 and MCF-7 cancer cell lines compared to CDSW, averaging ~75% and ~56%. The untargeted metabolomics analysis displayed an increase in secondary metabolites, with a total of 27 metabolites. LC-QTOF analysis of bioaccessible carbohydrate metabolites in FDSW revealed the presence of two phytochemical groups, alkaloids, and terpenoids. This study highlights the potential of FDSW for producing value-added bio-based products with desirable characteristics and health benefits.

Keywords: Date pomace; antioxidant; antidiabetics, ACE-inhibition, cytotoxicity.

5.2 Introduction

Date palm, *Phoenix dactylifera* L., is a traditional and economical crop planted in the Middle East and North Africa, where date fruit is a staple food (Oladzad et al., 2021). In 2021, the harvested area of the date palm reached 1.3 million hectares, producing about 9.6 million tonnes of date fruits worldwide, where 56% and 43% of this production came from Asia and Africa, respectively (FAOSTAT, 2021). Date fruits supply a significant source of soluble and insoluble dietary fibers, vitamins, and minerals, as well as a variety of bioactive compounds such as polyphenolic compounds, carotenoids, phytosterols, tocopherols, and tocotrienols (Fernández-López et al., 2022). FAO reported that the top 10 producers of dates in 2021 were Egypt, Saudi Arabia, Iran (Islamic Republic of), Algeria, Iraq, Pakistan, Sudan, Oman, United Arab Emirates, and Tunisia, respectively (FAOSTAT, 2021).

Usually, the date palm fruit is freshly consumed, while lower-quality dates are exploited to produce different food products such as syrup, jelly, juice, jam, and powder. Date residues, including date syrup waste (DSW), date seeds, and cull dates, result from date processing industries, and part of them are used as animal feed, while a large quantity has been discarded as waste (Oladzad et al., 2021).

DSW is a biomass of side-stream in the date processing industries of syrup or juice that remains after extraction (Rambabu et al., 2020). About 17-28% of DSW is generated daily from date juicing industries, mainly used as animal feed or discarded in open lands (Heidarinejad et al., 2018). Date waste has a high moisture content comprising the residual date pulp and remaining sugars, which are easily fermentable, leading to serious waste management issues (Oladzad et al., 2021) and increasing the risk of diseases by supporting the growth of pests, mice, and microorganisms. However, numerous studies have reported that DSW is rich in carotenoids, dietary fiber, phenolic compounds (e.g., flavonoids, phenolic acids, and quinones), and minerals (e.g., Ca, Fe, Mg, Cr, Mn) (Fernández-López et al., 2022). From a nutritional and economic perspective, DSW can be exploited to extract bioactive compounds to produce novel bio-based products via biorefinery technologies.

Proper and efficient strategies are required to reduce the adverse effects of DSW on the environment and to contribute to creating a sustainable economy in the date fruit industries. In this context, valorization of agro-food by-products through fermentation is well documented (Montero-Zamora et al., 2022; Sabater et al., 2020). Fermentation is a biological process carried out by microorganisms, primarily under anaerobic conditions, where they convert sugars into alcohols, carbon dioxide, and/or organic acids, resulting in the production of energy (Salminen et al., 2021). In microbial fermentation, the compatibility between the selected microorganism and the substrate assumes paramount importance in ensuring the success of the fermentation process (Sanchez & Demain, 2008). Utilizing food byproducts such as date syrup waste through fermentation is a great approach. This method releases a diverse range of bioactive compounds as primary and secondary metabolites, including vitamins, antibiotics, organic acids, antimicrobial peptides, folates, carbon dioxide, and alcohol. These compounds have therapeutic potential (Montero-Zamora et al., 2022). Because of its distinct composition, DSW (which has high sugar and low moisture content) is best fermented using yeasts. Among the potential probiotic yeasts that have considerable fermentation capabilities, *Pichia cecembensis* and *Issatchenkia orientalis* (*Pichia kudriavzevii*) have been identified, isolated, and characterized by Alkalbani et al. (2022). Assessing the health-promoting benefits of bio-treated food by-products is an intrinsic step to producing and offering these evaluated products on the market scale. Recently, several studies have reported the health-promoting benefits (e.g., antidiabetic and antioxidant activities, angiotensin-converting enzyme (ACE) inhibition, and antiproliferation) of fruit pomaces based on bioaccessible portions after in vitro digestion (Fernández-López et al., 2022). Furthermore, the UPLC-QTOF technique has been more recently used to analyze targeted and untargeted metabolomics of the bioaccessible portion to explore the metabolites and metabolic pathways (Ayyash et al., 2021a).

The adoption of the circular bio-economy concept by valorizing fruit by-products has garnered attention from the scientific community. Thus, the present study was performed on FDSW and CDSW, aiming to (1) analyze the chemical characteristics of FDSW and CDSW, including organic acids and sugars, volatile compounds, and phenolic compounds; (2) evaluate their health-promoting benefits comprising

antidiabetic, antioxidant, cytotoxicity, total phenolic compounds (TPC), proteolytic activity, and ACE inhibition, before and after in vitro digestion of bioaccessible portions; and (3) identify untargeted carbohydrate metabolites in the bioaccessible portions using UPLC-QTOF.

5.3 Materials and Methods

5.3.1 Chemicals

All chemicals utilized in this paper were sourced from Sigma-Aldrich (St. Louis, MO), unless otherwise specified. Yeast Extract– Peptone–Dextrose (YPD) broth (Himedia Laboratories Pvt ltd, Nashik, India), YPD agar (Himedia Laboratories Pvt ltd, Nashik, India), glycerol, peptone water, sodium hydroxide, hydrochloric acid, phosphoric acid, oxalic acid, citric acid, malic acid, lactic acid, acetic acid, propionic acid, sucrose, glucose, fructose, arabinose, nitroterephthalic acid, formic acid, p-hydroxybenzoic acid, gentisic acid, vanillic acid, gallic acid, (+)-taxifolin, iso ferulic acid, ferulic acid, quercetin, apigenin, myricetin, iso quercetin, protocatechuic acid, chlorogenic acid, p-coumaric acid, sinapic acid, caffeic acid, (+)-catechin, tyrosol, syringic acid, vanillin, rutin, luteolin, kaempferol, chrysoeriol, isorhamnetin, (-)-epicatechin, potassium chloride, potassium dihydrogen phosphate, sodium bicarbonate, magnesium dichloride hexahydrate, ammonium carbonate, calcium chloride dihydrate, corn starch, 3,5-dinitrosalicylic acid, sodium potassium tartrate, gallic acid, p-Nitrophenyl-α-D-glucopyranoside (pNPG), sodium carbonate, 1,1-diphenyl-2-picrylhydrazyl (DPPH), methanol, potassium persulphate, 2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid), Dulbecco's modified Eagle's medium (Gibco®, Invitrogen, Carlsbad, CA, USA), Fetal bovine serum (FBS), penicillin, streptomycin, Abcam Cell Cytotoxicity Assay Kit (ab112118, Cambridge, MA, USA), doxorubicin, sodium tetraborate (borax), sodium chloride, boric acid, N-Hippurly-His-Leu hydrate (HHL), ethyl acetate, sodium dodecyl sulphate, β-mercaptoethanol, Follin-Ciocalteu reagent, α-Glucosidase (100 UN), α-amylase from human saliva (1KU), pepsin, pancreatin, bile salts, angiotensin converting enzyme (ACE).

5.3.2 Yeast Culturing, Population, pH, and Titratable Acidity

Pichia cecembensis (*P. cecembensis*) and or *Issatchenkia orientalis* (*I. orientalis*), with accession numbers OK441068 and OK441071, respectively, were previously isolated from traditional fermented food sources and identified as potential probiotic yeasts (Alkalbani et al., 2022). Both yeasts were sub-cultured in YPD broth and stored in glycerol stocks (50% v/v) at -80ºC. YPD broth (Himedia Laboratories Pvt ltd, Nashik, India) was used to culture *P. cecembensis* and *I. orientalis*, separately, before being used for fermentation. The yeast population in FDSW and CDSW (control) was enumerated by serially diluting with 0.1% (w/v) peptone water and using the pour-plate technique. The plates (triplicates) were incubated aerobically at 25ºC for 6 days, and yeast populations were enumerated on YPD agar at 0, 3, 6 days. A calibrated digital Start-3100 pH meter (OHAUS Corporation, NJ, USA) was used to measure the pH value of FDSW and CDSW. Besides, samples were titrated with 0.01 N NaOH to determine the titratable acidity (TA%), which was expressed as a percentage of lactic acid.

5.3.3 Fermentation of Date Syrup Waste

DSW is a by-product of date syrup, consisting of cohesive, semi-moist biomass (~ 45%) that primarily contains the pulp and skins of date fruits, with a minor presence of residual fruit stalks. The DSW used in this study was derived from mixed date varieties and obtained from Al Barakah Dates Factory LLC (Dubai, UAE). DSW was diluted with distilled water at a 1:10 ratio before fermentation. The diluted DSW was inoculated with approximately 7.0 log CFU/mL (Appendix D.1) of *P. cecembensis* or *I. orientalis* and incubated at 25ºC for 6 days until pH reached 4.0 (Appendix D.1). The DSW without inoculation served as a control (CDSW).

5.3.4 Chemical Characteristics

5.3.4.1 Organic Acids and Sugars

Organic acids were analyzed using high-performance liquid chromatography (HPLC, Gradient solvent delivery system (Thermo Scientific Dionex Ultimate 3000 Rapid Separation LC system with Chromeleon (c) Dionex version 7.2.4.8179 software, Bremen, Germany)). The column used was a Supelcogel H 30 cm x 7.8 mm Cat #

59304-U at 25°C. The mobile phase was 85% (v/v) phosphoric acid (0.04 M H_3PO_4), and the flow rate was 0.4 mL/min. The PDA 3000 Dionex Photo Diode Array Detector (190–800 nm) was used for detection. The sample solution was transferred into an HPLC auto sampler vial, and 20 µl of the sample extract was injected in duplicate. The concentration of organic acids in the sample was calculated from the calibration curve using external standards.

A chromatographic analysis of sugars was performed with a SupelcoGel Ca+ column (300 × 7.8 mm; Supelco, Sigma-Aldrich, USA), at 85°C, in isocratic mode using deionized water as the mobile phase at 0.5 mL min^{-1}. Detection was performed using PDA at 195 nm.

5.2.4.2 Volatile Compounds

A 2 mL of FDSW and CDSW were centrifuged at 10,000 xg for 10 min at 4ºC to obtain the supernatant. The supernatant was then filtered using a 0.22-µm syringe filter. Volatile compounds were analyzed using headspace (HS) solid-phase microextraction (SPME) method, combined with carboxen/poly (dimethylsiloxane) fibre (85 µm coating, Supelco, Sigma-Aldrich, Barcelona, Spain), a gas chromatography (GC)-mass spectrometer (MS), and a flame ionization detector (FID). Filtered DSW samples (1 mL) were mixed with a saturated sodium chloride solution (1 mL), and 2 mL of the mixed solution was extracted with HS-SPME at 60°C for 50 min under a rotational speed of 250 rpm min^{-1}, in a 20-mL headspace vial. Volatile compounds were separated by a capillary column (60 m × 0.25 mm I.D., Agilent DB-FFAP, Santa Clara, CA, USA) coated with a 0.25-mm film thickness of polyethylene glycol modified with nitroterephthalic acid. Helium was used as the carrier gas, with a flow rate of 1.2 mL min^{-1}, and the temperature program increased from 50°C (5 min) to 230°C (30 min) at a rate of 5 °C/min. Volatiles were identified by comparing their mass spectra (MS) with NIST MS library, with a matching factor over 80.

5.2.4.3 Quantification of Phenolic Compounds

The phenolic compounds of FDSW and CDSW (control) samples were quantified using a Shimadzu U-HPLC system equipped with a reversed-phase analytical column of

2.1× 50 mm, 1.7-µm particle size (Waters Acquity UPLC BEH C18, Waters Corporation, Milford, USA). The column oven temperature was maintained at 45°C and the flow rate was set at 0.4 µl /min throughout the experiment. Water and acetonitrile containing 5% formic acid were used as mobile phases A and B, respectively. The injection volume was 2 µL of the prepared extract in section 5.2.4.2, with a run time of 20 min. The gradient profile began at 5% and increased to 20% B for 6 min, then increased to 70% B for 15 min, then increased to 100% B for 1 min before returning to the starting condition from 18-20 min. The effluents were measured at multiple wavelengths (260, 270, 280, 320, 340, and 370 nm). Phenolic compounds were identified by comparing their retention times and UV-Vis spectra with phenolic standards (p-hydroxybenzoic acid, gentisic acid, vanillic acid, gallic acid, (+)-taxifolin, iso ferulic acid, ferulic acid, quercetin, apigenin, myricetin, iso quercetin, protocatechuic acid, chlorogenic acid, p-coumaric acid, sinapic acid, caffeic acid, (+)-catechin, tyrosol, syringic acid, vanillin, rutin, luteolin, kaempferol, chrysoeriol, isorhamnetin, (-)-epicatechin).

5.3.5 In vitro Digestion by INFOGEST2.0 and Bioaccessible Portion

In vitro digestion was performed on FDSW and CDSW using the method described by Brodkorb et al. (2019), and the bioaccessible portion was conducted by following the method of Ayyash et al. (2021a). Briefly, 4.5 mL of FDSW and CDSW samples were subjected to in vitro oral digestion (amylase 75 U/mL, salivary fluid SSF, 0.3 M CaCl2, 2 min), gastric digestion (pepsin 2,000 U/mL, gastric juice SGF pH 3.0, 0.3 M CaCl2, 120 min), and intestinal digestion (pancreatin 100 U/mL, bile 10 mmol/L, duodenal juice SIF pH 7.0, 0.3 M CaCl2, 120 min). Before the start of the intestinal phase, a dialysis membrane (10 kDa MWCO, Thermofisher) filled with 37.5 mL of 0.5 M NaHCO3 solution was submerged in the digesta. The portion inside the dialysis membrane (10 kDa permeable) was described as bioaccessible and was stored at −20°C for further analysis.

5.3.6 Health-Promoting Indicators

The health-promoting properties (amylase and glucosidase inhibitions, antioxidants, ACE inhibition, cytotoxicity, TPC, OPA) of FDSW and CDSW samples

were evaluated before and after six days of fermentation, both in their undigested (using the extract prepared in Section 5.2.4.2) and *in vitro* digested states (bioaccessible portion in Section 5.2.5).

5.2.6.1 Starch Hydrolase Inhibition Activity

The α-amylase (human salivary, 1KU) and α-glucosidase (*S. cerevisiae,* 100UN) inhibition assays were carried out according to the method described by (Ng et al., 2020) for α-amylase and Ho et al. (2022) for α-glucosidase. The following equation was used to calculate the inhibition percentage:

$$\text{Inhibition } \% = \left(1 - \frac{Abssample - Absblank}{Abscontrol}\right) \times 100$$

where Abscontrol = absorbance of mixture without sample and Absblank = absorbance of the sample, enzyme, and buffer.

5.2.6.2 Antioxidant Capacity

The assays of radical scavenging rate by DPPH (1,1-diphenyl-2-picrylhydrazyl) and ABTS (2,2′-azino-bis(3-ethylbenzo- thiazoline-6-sulphonic acid) ($ABTS^{\bullet+}$)) were performed according to Ng & Kuppusamy (2019). The percentage of radical scavenging activity was calculated as follows:

$$\text{Scavening rate } \% = \left(\frac{Absblank - Abssample}{Absblank}\right) \times 100$$

5.2.6.3 Cytotoxicity Activities

The samples of the bioaccessible portion were assayed against Caco-2 and MCF-7 carcinoma cell lines according to the method detailed by Ayyash et al. (2021a). The following equation calculated the cytotoxicity percentage:

$$\text{Antiproferative activity } (\%) = \left[1 - \frac{Rsample - R0}{Rctrl - R0}\right] \times 100$$

Where, Rsample is the absorbance ratio of OD570/OD605 for the FDSW. Rctrl is the absorbance ratio of OD570/OD605 for CDSW (vehicle control). Ro is the averaged background (non-cell control) absorbance ratio of OD570/OD605.

5.2.6.4 Free Amino Acid Contents and TPC

The *O*-phthaldialdehyde (OPA) assay was conducted to estimate the total free amino acids as described in the method of Ayyash et al. (2021a). The TPC was measured using the Follin-Ciocalteu reagent method (Kashyap et al., 2022). Gallic acid served as the standard, and the results were presented in milligram gallic acid equivalent (mg GAE/g sample).

5.2.6.5 Angiotensin-Converting Enzyme (ACE) Inhibition

ACE-inhibition activity was assessed according to method described by Liu et al. (2018a). The ACE inhibition activity (%) was calculated as:

$$\text{ACE inhibition } \% = \left(1 - \frac{Ab - Aa}{Ab - Ac}\right) \times 100$$

Ab refers to the absorbance without adding the sample solution (buffer solution added instead of the sample), and *Aa* refers to the absorbance in the presence of ACE and the sample solution. *Ac* refers to the absorbance of the blank (HCl was added before the addition of ACE).

5.3.7 Untargeted Metabolomics and Carbohydrate Metabolomics Analyses

5.3.7.1 Untargeted Metabolomics

The bioaccessible portions of CDSW and FDSW fermented by *I. orientalis* on the 6th day were analyzed using UPLC-QTOF. The analysis utilized a Waters UPLC I-Class Plus (Waters, USA) tandem Q Exactive high-resolution mass spectrometer (Thermo Fisher Scientific, USA) to separate and identify metabolites. The chromatographic separation was carried out using a Waters ACQUITY UPLC BEH C18 column (1.7 μm, 2.1 mm × 100 mm, Waters, USA) with a column temperature of 45°C. The mobile phase was composed of 0.1% formic acid (A) and acetonitrile (B) in positive mode and 10 mM ammonium formate (A) and acetonitrile (B) in negative mode. For mass spectrometry, Q Exactive (Thermo Fisher Scientific, USA) was used for primary and secondary data acquisition. The equipment used in the experiment had a scanning range from 70 to 1050 m/z with a resolution of 70000. During the MS acquisitions, the AGC target was set to 3e6, and the resulting data were analyzed using Compound Discoverer 3.3 (Thermo

Fisher Scientific, USA) in combination with bmdb (BGI metabolome database), mzcloud database, and chemspider online database. The resulting data matrix contained information such as metabolite peak area and identification results. The table was further analyzed and processed using Compound Discoverer version v.3.3 with the parameters set to parent ion mass deviation < 5 ppm, mass deviation of fragmented ions < 10 ppm, and retention time deviation < 0.2 min, official Website: https://mycompounddiscoverer.com.

5.3.7.2 Carbohydrates Metabolomics Analysis

For metabolite extraction, 25 mg of tissues were weighed and extracted by directly adding 800 μL of precooled extraction reagent (methanol: acetonitrile: water (2:2:1, v/v/v)). Internal standards mix 1 (IS1) and internal standards mix 2 (IS2) were added for quality control of sample preparation. The samples were homogenized for 10 min, sonicated, and incubated at -20˚C for 1 hour before being centrifuged and the supernatant was then transferred for vacuum freeze drying. The metabolites were resuspended in 10% methanol and analyzed using LC-MS/MS with a 2D UPLC (waters, USA) and a tandem Q-Exactive mass spectrometer (Thermo Fisher Scientific, USA) with a heated electrospray ionization (HESI) source. The analysis was controlled by the Xcalibur 2.3 software program (Thermo Fisher Scientific, Waltham, MA, USA). The chromatographic separation and mass spectrometry were carried out as described in section 5.2.7.1.

5.3.8 Data Processing

The commercial software Progenesis QI (Waters, UK) and in-house metabolomics analysis process were used to conduct in-depth analysis of mass spectrometry data, with metabolite identification was based on HMDB (http://www.hmdb.ca/) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (https://www.genome.jp/ke gg/) databases. Principal component analysis (PCA) and heatmap analysis were performed. All analyses were performed by BGI Company (Shenzhen, China). The BMDB Library (BGI Metabolome Database), an in-house-developed database including retention time (RT), MS1 spectrum (MS1), and MS2 spectrum (MS2) of all standards, was employed. Key primary metabolites and metabolic

intermediates in the key metabolic pathways are covered, including carboxylic acids, amino acids, biogenic amines, polyamines, nucleotides, coenzymes and vitamins, monosaccharides and disaccharides, fatty acids, lipids, steroids, and hormones. HMDB, currently one of the most comprehensive databases of human metabolites, was also employed. The datasets cover the following three categories: a) chemical data, b) clinical data, and c) analytical biology and biochemical data. Both water-soluble and fat-soluble metabolites are included. In addition, the included information also provides links to other databases (e.g., KEGG, PubChem, etc.) for easy access. Three internal standards of d3-Leucine, 13C9-phenylalanine, d5-tryptophan, and 13C3-progesterone were employed for normalization using Probabilistic Quotient Normalization (PQN). Three internal standards of d3-Leucine, 13C9-phenylalanine, d5-tryptophan, and 13C3-progesterone were employed for normalization using Probabilistic Quotient Normalization (PQN).

5.3.9 Statistical Analysis

All results were expressed as the mean of three independent determinations ± standard deviation (SD), unless otherwise mentioned. The statistical mean difference among the samples was determined by analysis of variance (ANOVA) with Tukey post-test (Ng et al., 2020). PCA for variables and observations was conducted to estimate the structural correlation of the variables and to indicate the relationship between the fermented DSW and control. The statistical analyses were performed by XLSTAT software (Addinsoft, New York, NY, USA).

5.4 Result and Discussion

5.4.1 Yeast Population, pH, and Titratable Acidity

When microorganisms are used in food fermentation, their population must be maintained at viable levels throughout the fermentation period, enabling them to exert their full potential. The degree of fermentation is determined by the changes in pH values within the fermentation broth.

In the FDSW, the yeast strains (*P. cecembensis* and *I. orientalis*) grew well and maintained high populations, above 7.0 log CFU/mL over 6 days (Appendix D.1-A). By contrast, the CDSW (control) only showed one log CFU/mL during 0, 3, and 6 days.

These results indicate that two yeast strains were compatible with date syrup waste (DSW) medium. As illustrated in Appendix D.1-B, the pH of FDSW dropped significantly ($p < 0.05$) from an average of 5.26 to below 4.2 after 6 days of fermentation at 25℃. The pH of the control sample (CDSW) remained stable at an average pH of 5.4 during the fermentation period. The pH reduction in the date syrup waste fermented by *I. orientalis* was slightly faster than *P. cecembensis* ($p > 0.05$). In harmony with the pH value, the FDSW had an increase in the titratable acidity over six days ($p > 0.05$) (Appendix D.1-C). The changes in pH and titratable acidity may be attributed to the increased levels of organic acids produced by yeast.

5.4.2 Chemical Characteristics

5.4.2.1 Evolution of Organic Acids and Sugars Levels

The microbial activity in the fermentation process would lead to the metabolization of carbohydrate content in DSW, mostly into ethanol and CO2. It also produces or gives rise to various other compounds, including organic acids, aldehydes, alcohols, esters, and ketones. Along with volatiles and sugars, organic acids determine the quality of fruits. However, during fermentation, an appreciable rise occurs in the amount of certain organic acids, notably lactic acid and citric acid. The concentration of organic acids in the food is influenced by different variables, such as food matric, starter cultures, and the incubation temperature and duration (Pereira da Costa & Conte-Junior, 2015). Over time, the fermentation activity of indigenous or added starter cultures of microorganisms may lead to the gradual accumulation of these acids (Pereira da Costa & Conte-Junior, 2015; Ricke, 2003) which could affect the sensory quality and nutritional value of the food. In this study, an evaluation of the new profile of organic acids and sugars for FDSW and CDSW was conducted.

Table 8: Changes of organic acids (mg/100 g) in unfermented (control) and fermented date syrup waste.

Sample	Oxallic acid	Citric acid	Malic acid	Lactic acid	Acetic acid	Propionic acid
Non-fermented	51.4 ± 9.6	111.8 ± 23.2	234.8 ± 4.8	475.0 ± 72.1	128.7 ± 8.7	ND.
P. cecembensis	69.7 ± 12.8	192.4 ± 22.6	3327.9 ± 35.2	754.7 ± 74.1	189.5 ± 22.8	ND.
I. orientalis	23.0 ± 0.7	159.9 ± 15.8	3714.8 ± 46.0	1171 ± 116.8	309.2 ± 47.5	325.6 ± 46.8

ND.: no detection

Table 8 presents the quantification of the organic acids in CDSW and FDSW by two yeast strains after six days of fermentation. The amount of all examined organic acids (oxalic, citric, malic, lactic, acetic, and propionic acids) increased ($p < 0.05$) compared to the control sample (CDSW), except for oxalic acid in FDSW by *I. orientalis* and propionic acid, which was newly produced in FDSW by *P. cecembensis*. The amounts of malic and lactic acids in FDSW by *P. cecembensis* (3327.9, 754.7 mg/100 g) and by *I. orientalis* (3714.8, 1171 mg/100 g) were significantly higher ($p < 0.05$) than in the unfermented sample (234.8, 475 mg/100 g), respectively. This implies that the inoculation of yeast strains in the DSW remarkably enhanced the production of malic acid and lactic acid. The sample fermented by *P. cecembensis* formed a higher amount of citric acid than that of *I. orientalis* (192.4, 159.9 mg/ 100 g), respectively. Conversely, acetic acid was noted in the DSW fermented by *P. cecembensis* in a lower amount (189.5 mg/100 g) than that fermented by *I. orientalis* (309.2 mg/100 g).

The diversity of the organic acid content present in date fruits is a critical factor in taste variations among date fruits. In this regard, several researchers have found variations in the organic acids content in date fruits according to their varieties (Hazzouri et al., 2019; Kamal-Eldin & Ghnimi, 2018). Nevertheless, Kamal-Eldin & Ghnimi (2018) reported that malic acid is a fundamental acid with a different ratio in date fruits, which could be associated with taste differences.

In the current study, a high amount of lactic acid produced in FDSW by two yeast strains was primarily related to the sugar metabolism pathway via glycolysis. Moreover, our data showed a predominance of malic acid and an increase in citric acid production in the FDSW, which can be attributed to the oxidative pathway of the tricarboxylic acid

(TCA) cycle (Zelle et al., 2008), where malic acid and citric acid can be excreted. Similar findings regarding the predominance of malic acid in their studied date fruit were observed by Al-Farsi et al. (2005). The abundance of this acid in date fruit may give it a distinctive fruity, tart, smooth, and mellow taste (Al-Farsi et al., 2005). The total sugars content in CDSW was around 35.6 g/100 mL. However, sugars (sucrose, glucose, fructose, and arabinose) were not detected in significant amounts in FDSW, indicating their consumption by *P. cecembensis* and *I. orientalis*.

5.3.2.2 Volatile Compounds

During fermentation, microorganisms exhibit numerous metabolic activities and secrete a wide range of extracellular enzymes (Martinez, Bressani, Dias, Simão, & Schwan, 2019), which may lead to alterations in the volatile profiles. Accordingly, this alteration was evaluated, to assess the suitability of our two yeast strains for modifying the volatile profiles of FDSW.

%RPA of volatile compounds of chemical classes identified in DSW before and after fermentation are shown in Figure 20 A total of 42 volatiles were detected, consisting of 9 volatile acids, 6 alcohols, 8 esters, 4 phenols, 3 ketones, 4 aldehydes, and 7 volatiles compounds belonging to different chemical groups (Appendix D.2). As shown in Figure. 20, the growth and metabolism of two yeast strains, P. cecembensis and *I. orientalis*, in the DSW significantly affected the production of volatile compounds belonging to alcohols (~38% RPA and 18% RPA, respectively) compared to the unfermented sample (~10.7% RPA). Likewise, the generation of esters rose from ~34% in the control sample to ~53% RPA in the FDSW by *I. orientalis* and slightly decreased to ~27% RPA in FDSW by *P. cecembensis*. In the phenols class, the volatiles declined from ~41% RPA to ~ 23% and 19% RPA in FDSW, respectively. Compared to the unfermented sample (control), 9 new volatile compounds were detected, including 4-methyl-2-oxovaleric acid, isobutanol, isoamyl acetate, ethyl octanoate, ethyl decanoate, 2-acetyl-4-methylphenol, 2-methyl-3-thiolanone, butyrolactone and 4-methylbenzaldehyde. The production of these new compounds could modulate the aroma for the date syrup waste.

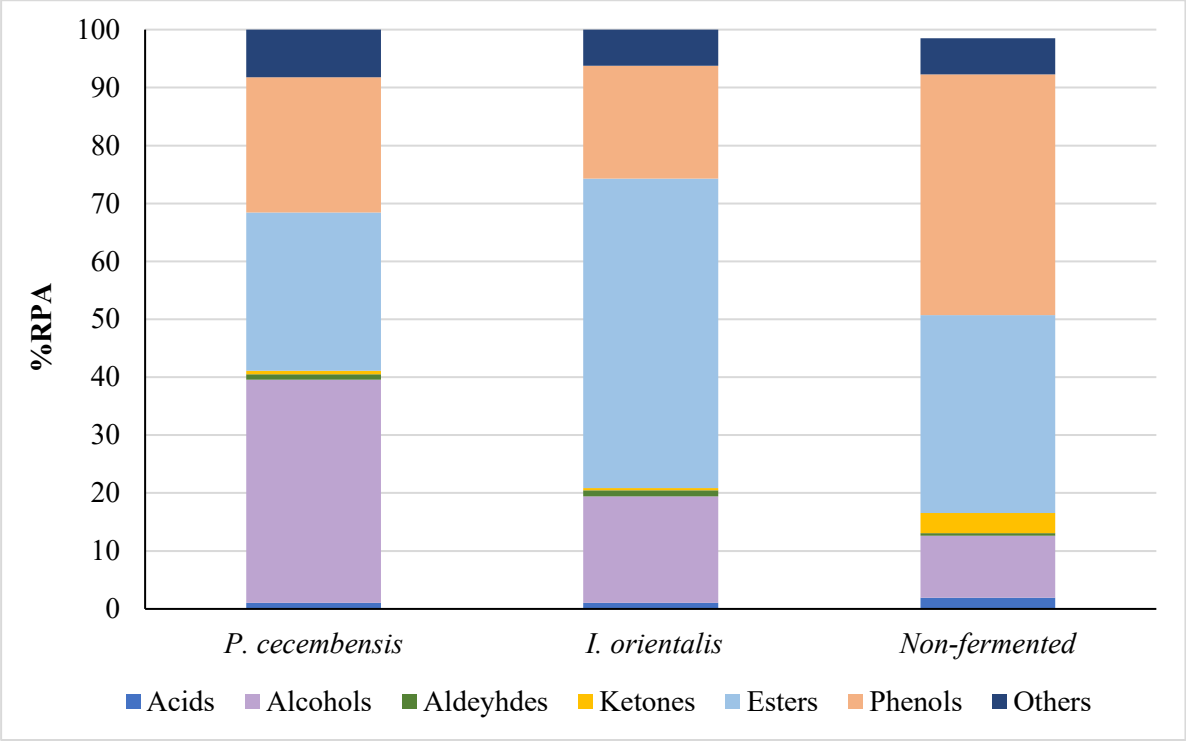

Figure 20: %Relative peak area (%RPA) pertains to the chemical families of volatile compounds detected in fermented date syrup waste and control.
Volatile compounds were analyzed using HS SPME method, combined with carboxen/poly (dimethylsiloxane) fibre, a GC-MS, and a flame ionization detector.

Acetic acid is the predominant compound in the acids group (Appendix D.2). De Vuyst & Leroy (2020) have shown that acetic acid is produced by yeast as a by-product of glycolysis, depending on yeast species and strains.

Different modes were observed for alcohols biosynthesis (Appendix D.2). Compared to the control, fermentation with *P. cecembensis* and *I. orientalis* led to the production of isobutanol, with *P. cecembensis* producing the highest level. It is likely that the amino acid valine was degraded to produce isobutanol in the yeast cytosol through the Ehrlich pathway (Wess et al., 2019). The levels of isoamyl alcohol and 2-phenylethanol had remarkably increased in fermented samples compared to the unfermented sample (control), with the highest level observed in the sample fermented by *P. cecembensis*. The higher content of alcohols like 2-phenylethanol and isoamyl alcohol can be produced from the Ehrlich pathway of sugar and amino acids (phenylalanine and leucine) metabolism in yeast fermentation (Hazelwood et al., 2008). Isobutanol, isoamyl alcohol, 1-hexanol, and 2-phenylethanol, which are present in the

samples, are known to convey alcoholic/solvent-like, banana/sweet/fruity, green/resin/flower, and rose aromas, respectively (Belviso et al., 2013). Overall, as expected, the total level of volatile alcohols in samples fermented with *P. cecembensis* and *I. orientalis* increased by ~12 and ~6 times, respectively, the value of CDSW.

As shown in Appendix D.2, an increasing trend was noted for esters after fermentation, except for tert-butyl formate, which decreased in the fermented samples by two yeast strains, as well as ethyl propanoate and ethyl octanoate, which were absent in the fermented samples by *P. cecembensis* and *I. orientalis*, respectively. Considerable differences were observed in the production of total esters after fermentation compared to CDSW, with the levels in FDSW increasing by more than 2.5- and 5-fold compared to those produced in CDSW, respectively. Ethyl acetate was shown to be the predominant volatile ester in FDSW. It is well known that the synthesis of volatile acetates (e.g., ethyl, isoamyl and phenethyl acetate) in yeast is performed via alcohol acetyltransferase, which catalyze the reaction of acetyl-CoA and alcohols (Shalit et al., 2001). Saerens et al. (2008) reported that ester production is greatly dependent on the yeast strain utilized, which could explain the differences in ester formation between the two yeast strains used in the current work.

In general, the use of two yeast strains to ferment DSW enhanced the production of phenols in this research by more than 1.5 times their total levels in CDSW. This study found phenols by 2,4-di-tert-butylphenol, 2-acetyl-4-methylphenol, t-butylhydroquinone, and guaiacol. All these phenols increased after fermentation by two yeast strains, except for 2-acetyl-4-methylphenol (Appendix D.2), which only formed in the fermented sample by *P. cecembensis*.

5.3.2.3 Phenolic Profile

The health benefits of phenolic compounds on the human body have been intensively discussed in scientific papers (Leonard et al., 2021). Logically, when compared with date fruits, the date syrup waste may have fewer polyphenols since it results from the date juice/syrup industry. Thus, some amounts of polyphenols are expected to transfer into the juice/syrup, and some of them may be exposed to enzymatic degradation during the processing stages. However, using yeast to ferment DSW could

compensate for this loss. Therefore, this paper screened the changes in the phenolics profile in FDSW and CDSW.

In our study, the U-HPLC system determined a total of ~ 70.0, 205.0, and 126.0 mg/kg of 19 phenolic compounds in CDSW (control), FDSW by *P. cecembensis*, and FDSW by *I. orientalis*, respectively (Table 9).

Table 9: Concentrations of phenolic compounds (mg/kg) of unfermented and fermented date syrup waste.

Compounds	***P. cecembensis***	***I. orientalis***	**Non-fermented**	**Group**
P-Hydroxybenzoic acid	10.2±1.82^{a}	8.2±1.48a^{b}	6.8±1.56^{b}	Phenolic acids
Gentisic acid	4.4±0.07^{a}	3.1±0.42^{b}	2.8±0.79^{c}	Phenolic acids
Vanillic acid	2.7±0.33^{b}	5.2±0.60^{a}	3.6±2.25^{c}	Phenolic acids
(+)-Taxifolin	3.3±0.35^{a}	2.6±0.35^{b}	2.5±0.70^{b}	Flavonoids
Isoquercetin	1.4±0.16^{a}	1.1±0.17a^{b}	0.6±0.14^{b}	Flavonoids
Ferulic acid	2.3±0.28^{a}	2.1±0.33a	1.6±0.32^{b}	Phenolic acids
Iso ferulic acid	1.2±0.18^{a}	1.1±0.09^{a}	1.1±0.15a	Phenolic acids
Protocatechuic acid	15.2±0.43^{a}	10.1±1.21^{b}	10.2±3.10^{b}	Phenolic acids
Gallic acid	18.4±1.99^{a}	11.6±1.05a^{b}	10.7±3.74^{b}	Phenolic acids
Chlorogenic acid	3.0±0.59b	3.0±0.31^{b}	4.9±1.31^{a}	Phenolic acids
p-Coumaric acid	1.6±0.26^{a}	1.1±0.09^{b}	1.4±0.31a^{b}	Phenolic acids
Sinapic acid	1.1±0.12^{a}	0.9±0.10^{b}	0.7±0.20^{c}	Phenolic acids
Caffeic acid	1.4±0.11^{a}	1.1±0.10^{b}	1.4±0.67^{a}	Phenolic acids
(+)-Catechin	10.3±1.15^{a}	9.5±0.71^{b}	7.0±1.93^{c}	Flavonoids
Tyrosol	36.8±0.44^{a}	21.1±1.84^{b}	8.4±2.22^{c}	Phenethyl alcohol
Syringic acid	14.2±0.27^{a}	5.7±0.74^{b}	4.6±2.32^{b}	Phenolic acids
Vanillin	2.8±0.27^{a}	1.2±0.04^{b}	1.4±0.86^{b}	Phenolic aldehyde
Rutin	1.5±0.26^{a}	0.9±0.13^{b}	0.3±0.22^{c}	Flavonoid
(-)-Epicatechin	73.8±8.85^{a}	36.4±3.40^{a}	N.D.	Flavonoids
T.A of phenolic acids	**75.4±6.45^{a}**	**53.3±6.52^{b}**	**49.8±16.72^{b}**	
T.A flavonoids	**90.2±10.77^{a}**	**50.6±4.76^{b}**	**10.3±2.99^{c}**	
T.A phenethyl alcohol	**36.8±0.44^{a}**	**21.1±1.84^{b}**	**8.4±2.22^{c}**	
T.A aldehyde	**2.8±0.27^{a}**	**1.2±0.04^{a}**	**1.4±0.86^{a}**	
Total phenols (mg/kg)	**205.2±17.94^{a}**	**126.0±13.14^{b}**	**69.9±22.79^{c}**	

ND.: no detection, T. A: total amount (mg/kg)
Values are means ± standard deviation of n=6

In general, yeast fermentation significantly increased the level of extracted phenolic compounds from the FDSW, especially gallic acid, protocatechuic acid, syringic acid, tyrosol, and (-)-epicatechin. FDSW fermented by *P. cecembensis* had a higher total amount of polyphenols than sample fermented with *I. orientalis*. Fermentation is known to alter the phenolic profile of plant foods by releasing certain phenolic monomers from complex forms (Leonard et al., 2021). Yeast enzymes, e.g. pectinases and β-glucosidase (β-glucoside glucohydrolase, EC 3.2.1.21), can contribute to the release of phenolic monomers (Zhang et al., 2021). The date fruit contains high levels of polymeric tanniferous melanin based on (-)-epicatechin (Alam et al., 2022). Interestingly, Table 9 reveals that (-)-epicatechin was newly generated during yeast fermentation in comparatively high amounts, accounting for 74 and 36 mg/kg in FDSW, respectively. The results of this study suggest that yeast fermentation was able to release (-)-epicatechin from its oligomeric proanthocyanidins and melanin (Cherif et al., 2021; Hammouda et al., 2013).

Besides (-)-epicatechin, the production of tyrosol, a phenylethanoid compound, significantly increased from 8.4 mg/kg in control to 36.8 and 21 kg/mg in FDSW, respectively. Tassoult et al. (2021) reported the detection of tyrosol in Algerian date pastes, however, the amount of this compound in our study was significantly higher than in others. This difference may be mainly attributed to yeast fermentation, where the ability of different yeast species to secrete tyrosol during fermentation has been reported by Roldán-López et al. (2022). Further, a considerable amount of hydroxybenzoic acid derivatives, including gallic acid (18.3 and 11.6 mg/kg) and protocatechuic acid (15.2 and 10.1 mg/kg) were detected in FDSW, with a higher amount in the sample fermented by *P. cecembensis*. Another hydroxybenzoic acid derivative detected in our work is syringic acid, accounting for 14.2 and 5.7 mg/kg in FDSW by *P. cecembensis* and *I. orientalis*, respectively. In agreement with our results, Al Juhaimi et al. (2020) assessed the different flesh date varieties for polyphenol constituents and identified syringic acid ranging from 0.8 to 13.6 mg/kg.

From a different perspective and considering the polyphenol classification, yeast fermentation greatly increased flavonoid production, resulting in approximately ~90 mg/kg and ~ 50 mg/kg in *P. cecembensis* and *I. orientalis* samples, respectively, as

compared to CDSW (~10 mg/kg). The phenolic acids content in *P. cecembensis* sample also increased by a total of 75 mg/kg, whereas *I. orientalis* samples remained relatively stable at ~ 53 mg/kg compared to the control (~ 50 mg/kg). As suggested by Haile & Kang (2019) and Moore et al. (2007), the rise in flavonoid and phenolic acids content could be attributed to an increase in acidic values during fermentation. This process releases insoluble bound flavonoid/phenolic acid components, making them more bioavailable.

Overall, the disparities in the Chemical properties (concentration and presence of organic acids, volatiles, and phenols) between this paper and the cited papers might be attributed to the effect of the yeast fermentation, differences in the food matrix between date syrup waste and date fruits, as well as other factors such as extraction methods, date maturity, and geographic origin of the date palm fruit.

5.4.3 Health-Promoting Parameters

Date fruits have recently gained considerable attention for their associated health benefits, such as antioxidant, antidiabetic, cytotoxic, and ACE-inhibitory activities. These benefits are largely attributed to the abundant dietary fiber and rich content of bioactive compounds found in date fruits (Fernández-López et al., 2022; Oladzad et al., 2021). Furthermore, the fermentation process was found to contribute to the release of bioactive compounds (Sabater et al., 2020). Therefore, it is expected that the fermentation process may lead to maximizing the health benefits of DSW. This study evaluated the health-promoting properties of CDSW and FDSW after in vitro digestion (bioaccessible portions), with the aim of efficiently utilizing food waste like fruit pomace.

In this paper, our results (Fig. 21(A-C) and Appendix D.3. A-B) indicate that there were observable differences in the health-promoting properties between the pre-*in vitro* digestion and bioaccessible portions (post *in vitro* digestion). As shown in Figure 21(A), the bioaccessible portions exhibited increased levels of α-amylase inhibition compared to undigested samples, with the maximum level observed in the *I. orientalis* sample at 53.9%. It has been suggested that polyphenols possess an inhibitory effect against α-amylase (Santana Andrade et al., 2022). The elevated polyphenol content in

FDSW, which could be attributed to yeast fermentation and subsequent increased degradation of bioactive compounds (Table 9), may offer a potential explanation for the greater α-amylase inhibition observed in FDSW before and after digestion, in comparison to CDSW. Figure 21(C) indicates that the percentages of ACE inhibition remained relatively stable at an average of ~ 30% for FDSW and ~ 27% for the control sample. Additionally, compared to CDSW, the average of the cytotoxicity activities of FDSW in the bioaccessible portion against colon and breast cancer cell lines considerably developed from 32.8% and 26.7%, respectively, to ~75.4% and ~56.5% for the FDSW samples, respectively. These upward/stable trends for inhibitions of α-amylase and ACE suggest an increase/maintenance in the concentration of related bioactive compounds, resulting from metabolized DSW by yeasts, during digestion.

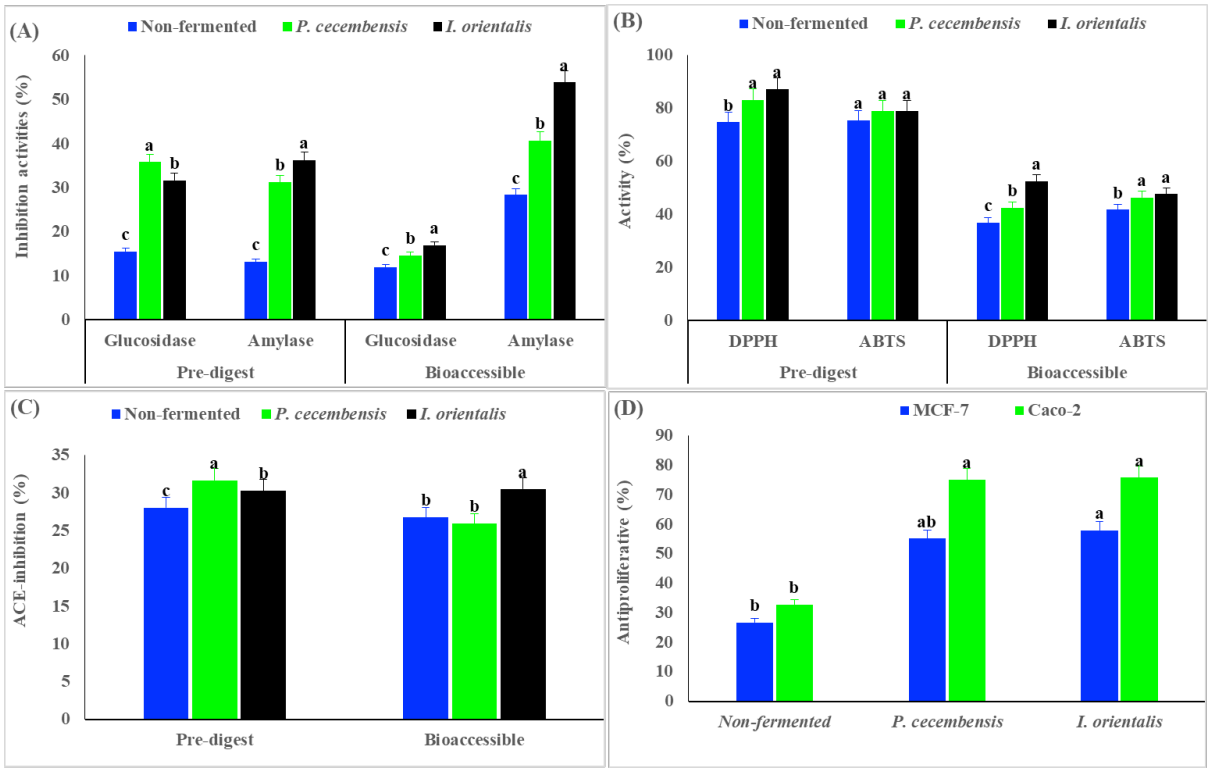

Figure 21: Evaluation of selected bioactivity of nonfermented and fermented date syrup waste for undigested samples and bioaccessible portions

Antidiabetic activities as measured by the α-amylase and α-glucosidase inhibitions (A), antioxidant activities (B), ACE inhibition (C) and cytotoxicity activity (D) of control and fermented date syrup waste by P. cecembensis and I. orientalis in the pre-digestion samples and bioaccessible portions.
Value = mean of triplicates. Standard deviation (SD) is displayed in error bars.

On the other hand, this work reveals that free amino acids, TPC, α-glucosidase inhibition, and antioxidant capacities of the bioaccessible portions had varying levels of reduction compared to the pre-digestion state. The TPC differed insignificantly ($p > 0.05$) between the three samples before and after the *in vitro* digestion. After digestion, the bioaccessible TPC content (Appendix D.3-A) showed a remarkable reduction ($p < 0.05$), decreasing from an average of 64.35 mg GAE/g for all three samples to ~10.0 mg GAE/g. Likewise, the absorbances of OPA decreased sharply ($p < 0.05$) in the bioaccessible portions compared to that before digestion, with values of 0.361, 0.372, and 0.233 for *P. cecembensis*, *I. orientalis*, and the control samples, respectively. The antioxidant capacities of CDSW and FDSW by two yeast strains, as measured by DPPH and ABTS, before and after (in bioaccessible portions) the *in vitro* digestion, are exhibited in Figure 21(B). In the bioaccessible portions, the DPPH scavenging rates dropped significantly ($p < 0.05$) to about half of their percentage before the in vitro digestion, with values of 42.5%, 52.4%, and 36.8% for *P. cecembensis*, *I. orientalis*, and control, respectively. Regarding the ABTS% (Figure 21(B)), the scavenging percentage before *in vitro* digestion was 78.9% for two fermented samples and 75.3% for control, whilst in the bioaccessible portion, these rates declined ($p < 0.05$) by ~ 40% in all treated samples.

The mechanisms underlying the decrease in these nutraceutical properties, which could be obtained from foods after digestion (bioaccessible portions), are multi-factorial. Digestive conditions and enzymes can directly degrade bioactive compounds during digestion, reducing these nutraceutical properties in the bioaccessible portions (Wang et al., 2021a). Besides, another explanation is that phenolic compounds are sensitive to interacting with certain dietary constituents, such as minerals, macromolecules, and dietary fiber, during the digestion process (Chen et al., 2016). This interaction can change the structural and chemical properties of nutrients, influencing their digestibility (Jakobek, 2015). Therefore, antioxidant capacities may be reduced after digestion due to the transformation of antioxidant phenolics into different structures with different chemical properties. Furthermore, polyphenols related to nutraceutical properties can be bound to the food matrix during digestion, reducing their bioaccessibility (Jakobek & Matić, 2019). In agreement with our findings, Fernández-Fernández et al. (2021)

reported a decline in the antioxidant capacity and TPC levels of the digested citrus pomaces. Contrary to our results, Santana Andrade et al. (2022) reported an improvement in the antioxidant capacity and TPC of fermented Brazilian fruits residues after *in vitro* digestion. However, the differences relative to our results may be ascribed to the differences in the structural modification of the bioactive compounds resulting from the in vitro digestion process and the metabolic activity during microbial fermentation process in different food matrices. Overall, in the bioaccessible portion, our findings indicate no significant differences between the two fermented samples, except for the inhibition of α-amylase and ACE, where the *I. orientalis* sample has significantly higher inhibitory effects than the *P. cecembensis* sample. However, the variations between the findings of the two yeast strains can be attributed to differences in their fermentation properties, such as the level of acid production. These variations result in differences in the degree of fermentation, leading to varying levels of released bioactive compounds responsible for health-promoting benefits. Besides, in general, this work shows that FDSW exhibited higher levels of measured assays (in bioaccessible portions) than CDSW, with the exception of stable levels observed for TPC and ACE inhibition. This suggests the efficient role of yeast fermentation in metabolizing bioactive compounds present in DSW. Additionally, the results imply that the bioactive compounds derived from FDSW by yeast strains could have multifunctional attributes.

5.4.4 Non-Targeted and Carbohydrate Metabolomics Analysis

5.4.4.1 Analysis of Non-Targeted Metabolites

Microbial fermentation has the ability to alter metabolites, producing new ones that could impact functional activity. The approach of untargeted metabolomics analysis allows for the characterization and identification of a broad range of metabolites without prior knowledge of their identities. This provides valuable insights into the chemical composition of FDSW compared to CDSW and its potential effects on human health. Due to its comparatively stronger antioxidant capacities compared to the *P. cecembensis* sample, the *I. orientalis* sample, along with the control, was chosen for this phase of the study. Figure 22(A) and 22(B) illustrate the PCA and heatmap, respectively, of the differential metabolites detected in bioaccessible portions of FDSW by *I. orientalis* and

control on the 6th day of fermentation. Figure 22(A) presents distinct dissimilarities between the control sample and the sample fermented by *I. orientalis*. The PCA results indicate that the metabolites detected in *I. orientalis* sample were significantly different from those in the control, with PC1 and PC2 accounting for 90.29% and 4.42% of the variance, respectively. The results of the PCA revealed that the fermentation process utilizing *I. orientalis* yielded discernible variations in metabolite profiles when compared to the control group. As demonstrated in Figure 22(B), unsupervised hierarchical clustering of the three experimental replicates revealed a distinct cluster. Additionally, the *I. orientalis* sample was distinctly clustered from the control sample in Figure 22(B), with bioaccessible metabolites categorized into two major groups (G1 and G2), demonstrating differences in metabolite concentrations. Compared to the control, the concentrations of metabolites in G1 and G2 categories were significantly altered in the *I. orientalis* sample. Furthermore, the heatmap shown in Figure 22(B) provides additional evidence that bolsters this conclusion by highlighting the differences in both the types and concentrations of metabolites present in the two samples. This finding suggests that the biological functions of *I. orientalis* during DSW fermentation may be associated with the biosynthesis of new bioactive compounds or the improvement of existing ones. Particularly, in the *I. orientalis* sample, the concentrations of metabolites in the G2 group increased while those in the G1 group decreased when compared to the control. This implies that *I. orientalis* probably metabolized a portion of the compounds in the G1 group during the fermentation of DSW while simultaneously amplifying those, in the G2 group.

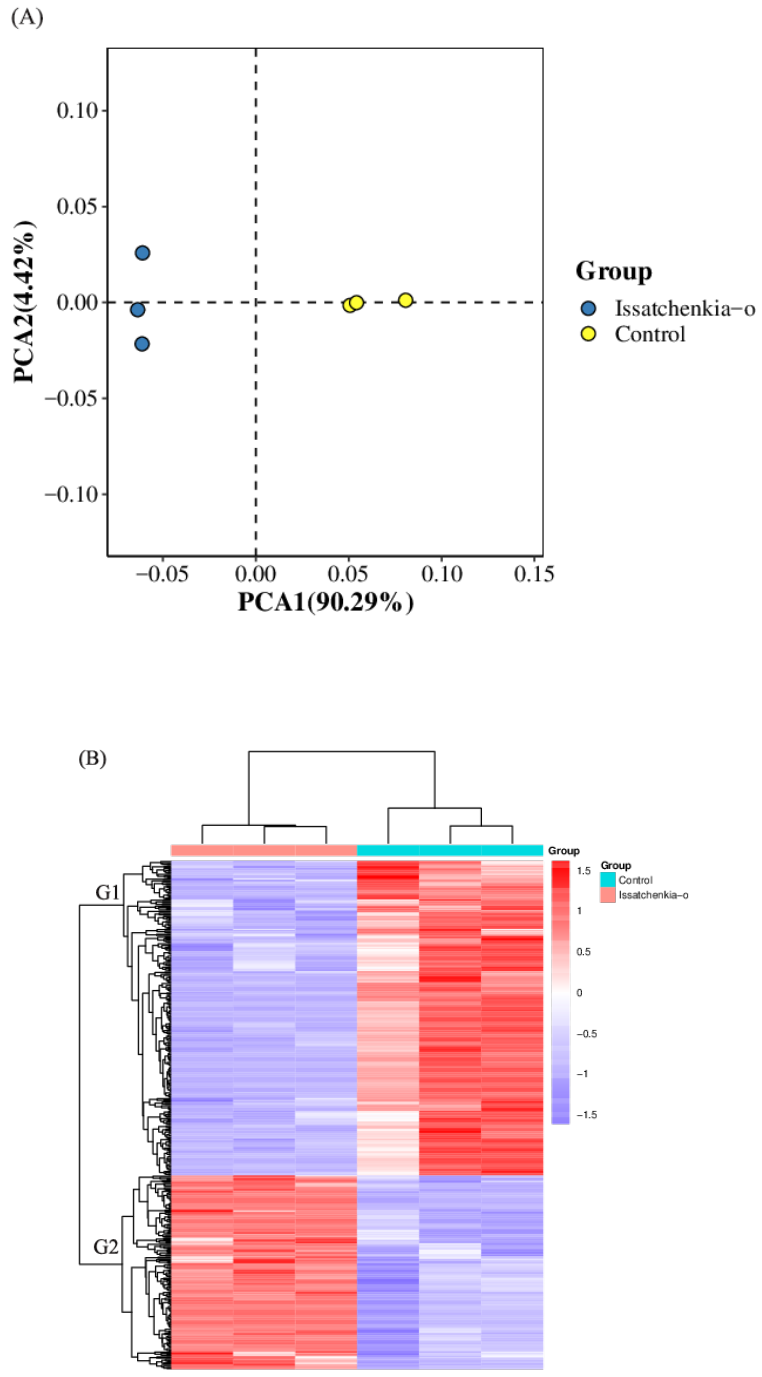

Figure 22: PCA score map (A) of the differential metabolites, and heatmap (B) depicts the metabolite concentration using a color gradient ranging from violet to red.

(the legend of the heatmap uses color references that are best understood by referring to the web version of the article).

5.3.4.2 Identification of Non-Targeted Metabolites

Conducting an analysis of metabolic pathway enrichment for differentially expressed metabolites using the KEGG database can unveil significant modifications in metabolic pathways, leading to an improved understanding of biological characteristics. In this work, metabolic pathways with a *p*-value of less than 0.05 were considered to be remarkably enriched with differential metabolites. As shown in Appendix D.5, bubble charts were generated to illustrate the top 10 metabolic pathways with the smallest *p*-values. The most prominent pathways identified in this analysis included biosynthesis of secondary metabolites and phenylalanine metabolism. Notably, the pathway for biosynthesis of secondary metabolites exhibited a significant increase in differential metabolites (Appendix D.5), with 27 metabolites identified in this pathway. Most of the 27 metabolites listed in (Appendix D.6), such as L-glutamic acid, succinate, L-arginine, L-methionine, valine, and L-isoleucine, exhibit therapeutic effects. Therefore, this study suggests a positive correlation between the presence of these metabolites and the functional activities observed in the *I. orientalis* sample. Differential metabolites were plotted on a volcano plot (Appendix D.4) to show upregulated, downregulated, and non-significant metabolites. The data in Appendix D.4 indicates that the total number of differentially identified metabolites between *I. orientalis* sample and control was 433, comprising 268 upregulated and 165 downregulated metabolites. Out of these 268 upregulated metabolites, particularly those identified in the metabolic pathway enrichment analysis (Appendix D.5 and Appendix D.6), it is highly probable that some of them are responsible for the observed bioactive activity in the *I. orientalis* sample. In support of this proposition, the data of volcano plot and Appendix D.5 showed that L-glutamic acid, succinate, L-arginine, valine, and L-isoleucine were among the upregulated metabolites. In the study conducted by (Fadimu et al., 2022), they highlighted the significant role of isoleucine, glutamic acid, and arginine in the inhibition of α-amylase activity. This finding may explain the superiority of the *I. orientalis* sample in α-amylase inhibition and also cytotoxicity activity observed in the investigation of health-promoting parameters in this study (Figure 21).

5.3.4.3 Identification of Carbohydrate Metabolites

Date fruits serve as an excellent source of quick energy, primarily due to their high carbohydrate content, which typically ranges from 70% to 80% (Tang et al., 2013). The yeast fermentation would bring about significant changes in carbohydrate metabolism present in DSW. To assess these changes in the bioaccessible portion of FDSW and CDSW, carbohydrate metabolites have been identified in this study. Figure 23 displays the results of carbohydrate metabolism (Figure 23(A)) and the analysis of the pathways linked with differentially regulated metabolites by using KEGG databases (Figure 23(B)). The differential metabolites, between *I. orientalis* sample and control, were categorized into phytochemical compounds, lipids, compounds with biological roles, and others (uncategorized) in Figure 23(A). The major classes of differential metabolites identified were terpenoids (2), lipids (6), amino acids, peptides, and analogs (6), benzene and derivatives (3), amines (3), and organic acids (2). Figure 23(A) results showed that the bioaccessible portions contained various compounds with biological roles, such as amino acids, benzene, antibiotics, peptides, amines, and organic acids. In addition, the bioaccessible portions also contained phytochemical compounds such as alkaloids and terpenoids, which are well-known for their anti-inflammatory, antioxidant, antidiabetic, and anti-proliferative effects (Liu et al., 2018a). Based on the KEGG database, the primary metabolism pathways of carbohydrates were global and overview maps (8), lipid metabolism (5), metabolism of terpenoids and polyketides (2), and amino acid metabolism (2), as illustrated in Figure 23(B). These findings strongly indicate that after fermentation by *I. orientalis*, DSW is likely to experience a substantial enhancement in its functionalities, which aligns with the health-promoting indicators found in this paper.

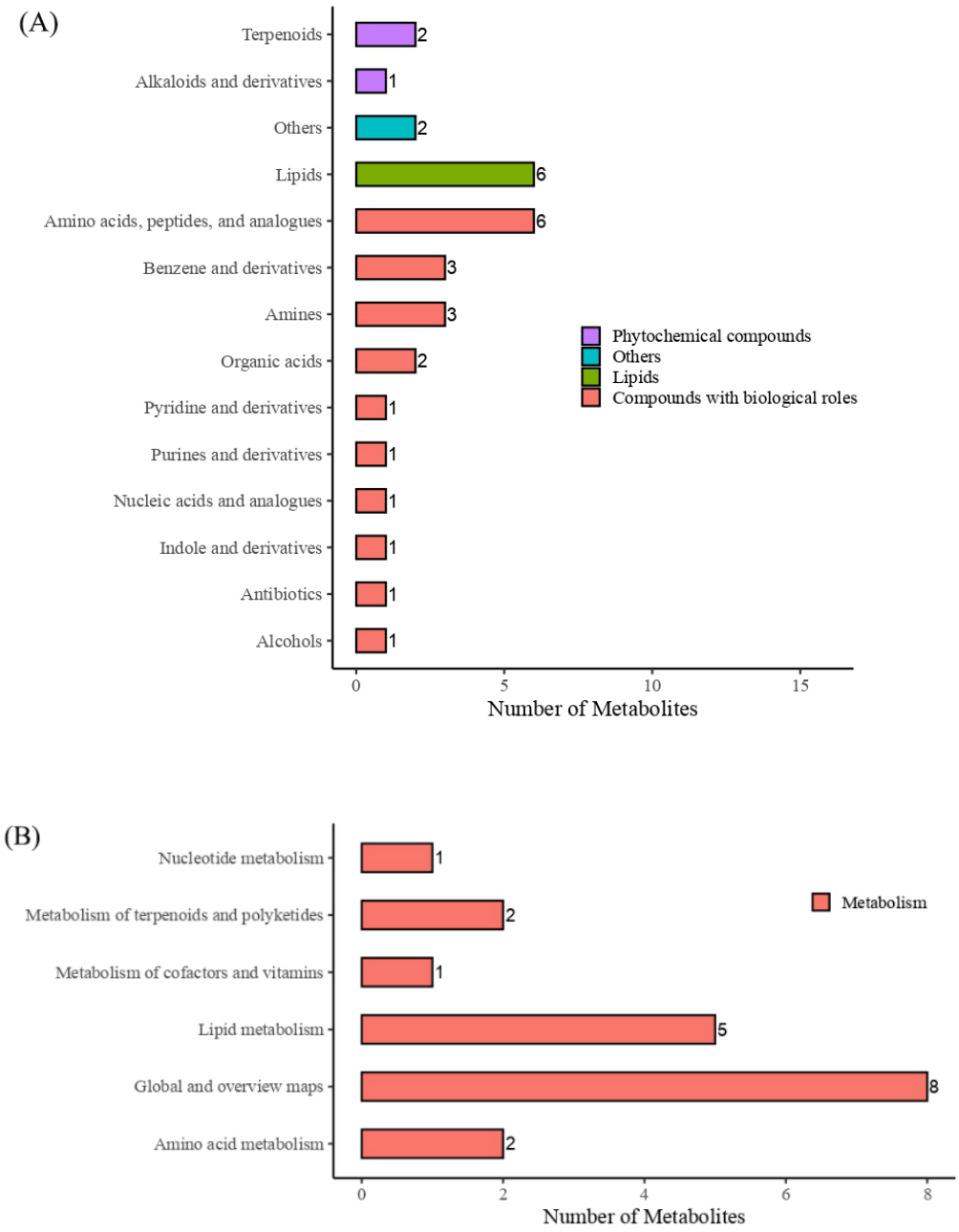

Figure 23: Identification of differential carbohydrate metabolites (A) between the bioaccessible portions of the *I. orientalis* sample and the control, and (B) elucidation of their involvement in KEGG pathway.

5.5 Conclusions

Fermentation with potential probiotic yeast enriches the health benefits of DSW, improving its chemical composition and potential effects. The two yeast strains had a significant impact on the fermentation of DSW by producing higher levels of lactic and acetic acids compared to the control group. Different strains of yeast resulted in varying levels of volatiles especially in alcohol and ester compounds, emphasizing the importance of selecting an appropriate fermentation agent to modulate the chemical composition of volatile compounds in DSW. Fermentation also generally increased the content of phenols in FDSW, which may improve shelf life, resistance to microbial growth, and sensory characteristics, compared to unfermented DSW. Moreover, two yeast strains were found to produce (-)-epicatechin at relatively high levels. Further targeted metabolomic analysis is needed to explore the mechanism and pathway of (-)-epicatechin production. To confirm the effectiveness of FDSW in metabolism, it is crucial to assess its bioaccessibility. The utilization of yeast species to ferment DSW can boost its health benefits, including amylase inhibition and cytotoxicity, which can provide additional prospects to use date by-products. KEGG pathway found that the biosynthesis of secondary metabolites was the most enriched metabolic pathway, including 6 functional amino acids, while 20 compounds with biological roles have been identified within the carbohydrate metabolites.

Author Contributions

N.A., writing—original draft, investigation, data curation, formal analysis; T.M.O., A.N.O., A.A.A.-N., S.-Q.L., R.S.O., writing—review and editing; writing—review and editing; M.M.A., conceptualization, writing—original draft, funding, supervising, writing—review and editing, supervision. All authors have read and agreed to the published version of the manuscript.

Data Availability

The authors declare that the data supporting the findings of this study are available within the paper and supplementary information files. The data also can be available from the corresponding author upon reasonable request.

Conflicts of Interest: The authors declare no conflict of interest

Chapter 6: General Discussion

The characterization of new probiotic candidates needs to follow the criteria established by the Food and Agriculture Organization/World Health Organization (FAO/WHO) in 2002. In this dissertation, a low acidic medium pH of 2.5 at 37°C was used as preliminary probiotic investigation in yeast isolates. Varying levels of survivability at low pH were observed in a total of 105 yeast isolates (Figure 24), ranging from 0.0% to 100%. Among them, 45 isolates that showed significant acid tolerance were selected for further investigation on their resistance to *in vitro* digestion conditions and bile salts.

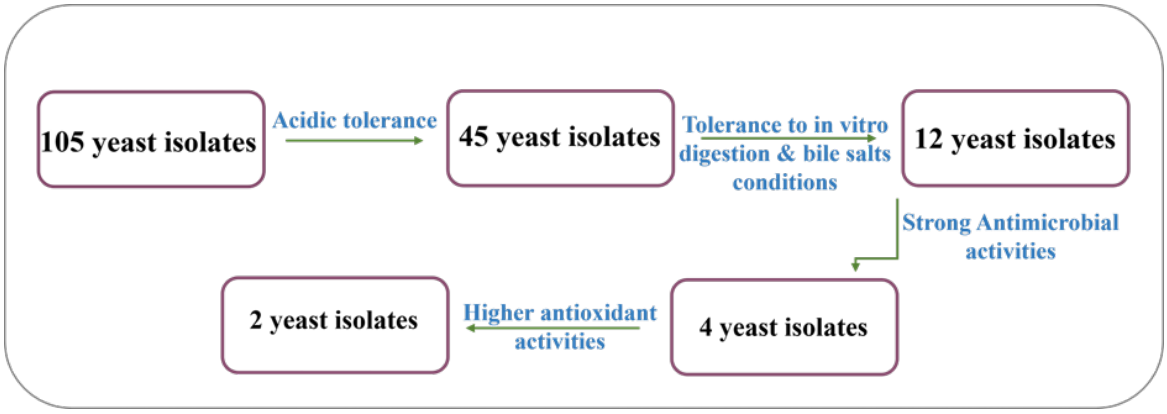

Figure 24: The steps and criteria for selecting potential probiotic yeast throughout all research stages.

Generally, adjustment of yeast cell walls and activation of stress response pathways enable yeast to resist strong inorganic acids in GIT conditions (Kapteyn et al., 2001; Lucena et al., 2020) . The ability to withstand the harsh conditions of the gastrointestinal tract is a crucial factor for microorganisms to be considered probiotic. This includes the capacity to endure the acidic nature of gastric juice, such as pepsin, as well as the alkaline pancreatic enzymes, lysozyme, and bile salts, all at the normal body temperature (Gut et al., 2018). All yeast isolates experienced reduced growth ($p < 0.05$) under *in vitro* digestion conditions, with reductions ranging from approximately 0.7 to 2.1 Log_{10}. Bile salt tolerance increased in yeast isolates with longer incubation periods, ranging from 69.2% to 91.1% after 24 hours. Twelve isolates with high survivability in *in vitro* digestion conditions were selected according to their varying isolated sources for

subsequent investigations. All selected yeast strains demonstrated the ability to remove cholesterol within a varied range and hydrolyze the tested bile salts. Furthermore, the selected isolates exhibited heat resistance, hydrophobicity, strong coaggregation, autoaggregation after 24 hours, robust antimicrobial activity, and EPS production. Overall, 9 out of 12 yeast isolates were sensitive or moderately sensitive to all the investigated antibiotics. Our antimicrobial activity results are in contrast to those of Amorim et al. (2018) because no antimicrobial activity was exhibited by their tested yeast isolates. However, the results obtained by Hossain et al. (2020) coincide with the current study. The identification of the 12 yeast isolates was determined by sequencing the ITS/5.8S ribosomal DNA. Out of 12 isolates, *C. albicans* was identified in one isolate, *S. cerevisiae* in another isolate, while the remaining 10 isolates were identified as *P. kudriavzevii.*

DPP is often regarded as a valuable biomass side-stream due to its composition, which can include diverse bioactive compounds (Fernández-López et al., 2022; Oladzad et al., 2021; Struck & Rohm, 2020). Consequently, DPP can be exploited to extract bioactive compounds and produce innovative bio-based products using biorefinery technologies. In this context, the fermentation-based valorization of agro-food by-products is extensively documented (Montero-Zamora et al., 2022; Sabater et al., 2020).

In this dissertation, 4 yeast isolates (Figure 24) with stronger antimicrobial activities and antibiotic sensitivity were selected to ferment DPP at 25°C for 6 days. After fermentation with selected yeast isolates, our data showed a predominance of malic acid and an increase in citric acid production in the fermented DPP, which can be attributed to the oxidative pathway of the tricarboxylic acid (TCA) cycle (Zelle et al., 2008), where malic acid and citric acid can be excreted. Kamal-Eldin & Ghnimi (2018) reported that malic acid is a fundamental acid with a different ratio in date fruits, which could be associated with taste differences. Forty-two volatile compounds were determined in DPP, and 9 new volatile compounds were detected in the fermented DPP. Varying levels of volatiles were observed due to different yeast strains, particularly in alcohol and ester compounds. For instance, alcohols were the predominant group in fermented samples by *P. cecembensis* and *S. cerevisiae*, whereas esters were the primary volatiles in fermented samples by *I. orientalis* and *P. kudriavzevii*. Ethyl acetate was

shown to be the main volatile ester in fermented DPP. It is well-known that the synthesis of volatile acetates (such as ethyl, isoamyl, and phenethyl acetate) in yeast is performed via alcohol acetyltransferase, which catalyzes the reaction between acetyl-CoA and alcohols (Shalit et al., 2001). Twenty phenolic compounds were identified in DPP, whereas (-)-epicatechin, tyrosol, and gallic acid were the significant phenols in fermented DPP. Interestingly, (-)-epicatechin was newly generated during yeast fermentation in comparatively high amounts. The results of this study suggest that yeast fermentation was able to release (-)-epicatechin from its oligomeric proanthocyanidins and melanin (Hammouda et al., 2013).

The results of health promoting indicators indicate that the bioaccessible portions exhibited increased levels of α-amylase inhibition, while the percentages of ACE inhibition remained relatively stable. Additionally, the cytotoxicity activities of fermented DPP were observed to have developed against colon and breast cancer cell lines when compared to the control. These upward/stable trends suggest an increase/maintenance in the concentration of related bioactive compounds during digestion. On the other hand, this work reveals that OPA, TPC, α-glucosidase inhibition, and antioxidant capacities of the bioaccessible portions had varying levels of reduction compared to the pre-digestion state. The mechanisms underlying the decrease in these nutraceutical properties, which could be obtained from foods after digestion (bioaccessible portions), are multi-factorial. Digestive conditions and enzymes can directly degrade bioactive compounds during digestion, reducing these nutraceutical properties in the bioaccessible portions (Wang et al., 2021a). Another explanation is that phenolic compounds are sensitive to interacting with specific dietary constituents, such as minerals, macromolecules, and dietary fiber, during the digestion process, thereby influencing their digestibility (Chen et al., 2016; Jakobek, 2015). Contrary to our results, Santana Andrade et al. (2022) reported an improvement in the antioxidant capacity and TPC of fermented Brazilian fruits residues after *in vitro* digestion. However, the differences relative to our results may be ascribed to the differences in the structural modification of the bioactive compounds resulting from the *in vitro* digestion process and the metabolic activity during microbial fermentation process in different food matrices. Generally, in this work, fermented DPP showed higher levels of measured

assays (in bioaccessible portions) than non-fermented samples, except for TPC and ACE inhibition, where the levels were almost stable, indicating the efficient role of yeast fermentation in metabolizing bioactive compounds present in DPP.

Due to the relatively superior antioxidant activities of the *I. orientalis* and *P. kudriavzevii* samples (Figure 24) compared to the other two samples of yeast strains, they have been selected, along with the non-fermented DPP (control), for untargeted metabolomics analysis. Conducting an analysis of metabolic pathway enrichment for differentially expressed metabolites using the KEGG database can reveal significant modifications in metabolic pathways, thus enhancing our understanding of biological characteristics. The differential metabolites between the *I. orientalis* sample and the control reveals that the most prominent pathway was biosynthesis of secondary metabolites, with 27 metabolites (such as succinate and L-arginine). Succinate is known to possess the ability to stimulate vascular tone, increase blood flow, improve age-related degenerative changes, and enhance antioxidant capacity (Chen et al., 2015), while the functional amino acid L-arginine is crucial in the cardiovascular system (Li et al., 2022).

Regarding the analysis of differential metabolites between the *P. kudriavzevii* sample and the control, C5-branched dibasic acid metabolism was the most notable pathway, with four metabolites identified in this pathway: itaconate, L-glutamic acid, oxoglutaic acid, and (+/-)-2-hydroxyglutaric acid. These four metabolites have been described to provide health-promoting benefits. Itaconate is known for its anti-immune and anti-inflammatory properties, L-glutamic acid acts as an anti-cancer agent, and oxoglutaric acid serves as a booster for collagen production (Dutta et al., 2013; Mills et al., 2018; Zhou et al., 2021). Moreover, the LC-QTOF analysis of bioaccessible carbohydrate metabolites in the fermented DPP by *I. orientalis* revealed the presence of two phytochemical groups, alkaloids and terpenoids. Alkaloids have been found to inhibit the topoisomerase enzyme, leading to DNA replication arrest and cell death (Dhyani et al., 2022).While the carbohydrate metabolites in the *P. kudriavzevii* sample unveiled the presence of a cluster of phytochemicals, encompassing three terpenoid metabolites. Triterpenoids have demonstrated diverse biological properties, including antiulcer, hepatoprotective, antiviral, antiatherosclerotic, and cholesterol-lowering

effects (Akihisa et al., 2001; Szakiel et al., 2012). In general, the metabolomics findings signify a substantial enhancement in the functions of DPP following fermentation.

Chapter 7: Conclusion and Future Perspectives

7.1 Conclusions, Limitations, and Suggestions

This dissertation aimed to maximize food waste utilization by providing insights into biological activities, phenolic and volatile compounds, untargeted metabolomics, and carbohydrate analysis after *in vitro* digestion of date pomace fermented with potential probiotic yeast. Selected yeast strains isolated from fermented dairy and non-dairy products exhibited probiotic characteristics. The probiotic yeast candidates exhibited exceptional survival rates following *in vitro* digestion, with the highest resistance resulting in a reduction of 0.7 Logs. Four promising yeast strains were used to ferment DPP, resulting in higher levels of lactic and acetic acids in the fermented samples compared to the control. The use of different yeast strains led to varied levels of volatile compounds, particularly alcohol and ester compounds. This highlights the importance of selecting the appropriate fermentation agent to control the chemical composition of volatile compounds in fermented DPP. Fermentation generally increased the phenolic content of fermented DPP, and four yeast strains were found to produce high levels of (-)-epicatechin, a compound not detected in the control sample. However, further targeted metabolomic analysis is necessary to understand the mechanism and pathway of (-)-epicatechin production. Further studies are required to investigate the safety of DPP on animals, to screen the synthesis of unhealthy compounds such as ethyl carbamate, and to evaluate the effects of anti-nutritional factors such as catechins. The bioaccessibility analysis revealed that the use of yeast species to ferment DPP can enhance its health benefits, including amylase inhibition and cytotoxicity. However, the scope of this study was limited due to a lack of availability for further investigation. Overall, the untargeted metabolomics results suggest a significant improvement in the performance of DPP after undergoing fermentation, indicating the potential for using selected yeast strains to enhance the quality and functionality of fermented products.

7.2 Research Implications

The dissertation found that novel yeast strains can ferment DPP and control the organic acids and chemical composition of volatile compounds. Yeast fermentation also results in a higher phenolic content, which could extend the shelf life, restrict microbial growth, and enhance the sensory qualities of the products. The study identified four yeast strains that could be used as cell factories in industrial biotechnology, as they produce high levels of (-)-epicatechin, a compound absent in the control sample. Fermenting DPP with yeast strains can improve amylase inhibition and cytotoxicity, providing more opportunities to use date by-products. The research can promote the development of sustainable and functional food products by using food waste.

7.3 Future Perspectives

The results of this dissertation can serve as a basis for future research on maximizing food waste utilization and improving fermented products. It would be useful to investigate how selected yeast strains can be used for industrial biotechnology and to analyze the chemical changes that occur during fermentation by conducting targeted metabolomic analysis to understand their effect on sensory properties and shelf life. Additionally, exploring the potential of using fermented DPP as a functional food ingredient through *in vivo* studies would be valuable.

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Appendix A.1: The survivability of selected yeast strains undergo in-vitro gastric conditions

Genus	Species	Survival Mean	Min.	Max.	C.	Reference
Aureobasidium	*Aureobasidium pullulans*	35.55	35.55	35.55	1	(Bonatsou et al., 2015)
		89.38	84.50	97.60	6	(Bonatsou et al., 2018)
Candida	*Candida adriatica*	23.4	16.60	30.20	2	(Zullo and Ciafardini, 2019)
	Candida boidinii	29.79	4.19	55.38	2	(Porru et al., 2018)
		16.16	16.16	16.16	1	(Oliveira et al., 2017)
		90.12	85.50	95.20	6	(Bonatsou et al., 2018)
	Candida diddensiae	65.67	64.94	66.40	2	(Porru et al., 2018)
		2.4	01.78	3.01	2	(Zullo and Ciafardini, 2019)
		81.73	77.30	88.20	3	(Bonatsou et al., 2018)
	Candida molendinolei	73.06	68.6	80.70	7	(Bonatsou et al., 2018)
	Candida naeodendra	86.70	81.40	92.00	2	(Stamatoula Bonatsou et al., 2018)
	Candida norvegica	49.75	49.75	49.75	1	(Oliveira et al., 2017)
	Candida silvae	33.25	33.25	33.25	1	(Bonatsou et al., 2015)
	Candida tropicalis	133.7	72.65	194.76	2	(Oliveira et al., 2017)
Citeromyces	*Citeromyces matrinensis*	86.9	86.90	86.90	1	(Stamatoula Bonatsou et al., 2018)
Cystofilobasidium	*Cystofilobasidium bisporidii*	94.8	94.80	94.8	1	(Stamatoula Bonatsou et al., 2018)
Debaryomyces	*Debaryomyces hansenii*	40.71	40.71	40.71	1	(Oliveira et al., 2017)
		90.37	90.37	90.37	1	(Bonatsou et al., 2015)
Metschnikowia	*Metschnikowia pulcherrima*	75.01	75.01	75.01	1	(Bonatsou et al., 2015)
		96.43	95.70	97.50	3	(Bonatsou et al., 2018)

pH.2, pepsin 0.0133g/L, 2.5 h, 37°C, buffer: NaCl (2.05 g/L), KH2PO4 (0.60 g/ L), CaCl2 (0.11 g/L) and KCl (0.37 g/L). Min.: minimum; Max.: maximum; C.: count

Appendix A.1: The survivability of selected yeast strains undergo in-vitro gastric conditions (Continued)

Genus	Species	Survival Mean	Min.	Max.	C.	Reference
Nakazawaea	*Nakazawaea molendini-olei*	15.43	15.43	15.43	1	(Porru et al., 2018)
		21.11	03.80	38.41	2	(Zullo and Ciafardini, 2019)
	Nakazawaea wickerhamii	75.86	75.86	75.86	1	(Zullo and Ciafardini, 2019)
Pichia	*Pichia guilliermondii*	179.43	179.43	179.43	1	(Oliveira et al., 2017)
		492.05	492.05	492.05	1	(Bonatsou et al., 2015)
		93.85	91.30	96.40	2	(Bonatsou et al., 2018)
	Pichia kluyveri	87.77	87.77	87.77	1	(Bonatsou et al., 2015)
		83.50	78.70	88.30	2	(Bonatsou et al., 2018)
	Pichia manshurica	29.63	29.63	29.63	1	(Oliveira et al., 2017)
		76.96	76.96	76.96	1	(Bonatsou et al., 2015)
		91.87	90.00	92.90	3	(Bonatsou et al., 2018)
	Pichia membranifaciens	60.44	60.44	60.44	1	(Bonatsou et al., 2015)
Rhodotorula	*Rhodotorula diobovatum*	47.54	47.54	47.54	1	(Bonatsou et al., 2015)
	Rhodotorula glutinis	86.37	75.70	91.70	3	(Stamatoula Bonatsou et al., 2018)
	Rhodotorula mucilaginosa	31.33	31.33	31.33	1	(Bonatsou et al., 2015)
		82.70	82.70	82.70	1	(Bonatsou et al., 2018)
Saccharomyces	*Saccharomyces boulardii*	95.88	95.88	95.88	1	(Porru et al., 2018)
		44.43	44.43	44.43	1	(Oliveira et al., 2017)
		66.06	66.06	66.06	1	(Zullo and Ciafardini, 2019)
	Saccharomyces cerevisiae	79.68	79.68	79.68	1	(Porru et al., 2018)
		21.16	3.16	39.15	2	(Oliveira et al., 2017)
		51.02	51.02	51.02	1	(S Bonatsou et al., 2015)
		93.03	89.90	98.80	6	(Bonatsou et al., 2018)

Appendix A.1: The survivability of selected yeast strains undergo in-vitro gastric conditions (Continued)

Genus	Species	Survival Mean	Min.	Max.	C.	Reference
Wickerhamomyces	*Wickerhamomyces anomalus*	49.15	49.15	49.15	1	(Porru et al., 2018)
		40.48	40.48	40.48	1	(Bonatsou et al., 2015)
		33.95	17.78	50.12	2	(Zullo and Ciafardini, 2019)
Yamadazyma	*Yamadazyma terventina*	17.91	10.12	25.7	2	(Zullo and Ciafardini, 2019)
Zygoascus	*Zygoascus hellenicus*	93.87	91.20	97.7	3	(Bonatsou et al., 2018)
Zygosaccharomyces	*Zygosaccharomyces mrakii*	19.54	19.54	19.54	1	(Porru et al., 2018)

pH.2, pepsin 0.0133g/L, 2.5 h, 37°C, buffer: NaCl (2.05 g/L), KH2PO4 (0.60 g/ L), CaCl2 (0.11 g/L) and KCl (0.37 g/L). Min.: minimum; Max.: maximum; C.: count

Appendix A.2: The survivability of selected yeast strains undergo in-vitro intestinal conditions

Genus	Species	Survival	Minimum	Maximum	Count	Reference
Aureobasidium	*Aureobasidium pullulans*	0	0	0	1	(Bonatsou et al., 2015)
		40.85	0	91.4	6	(Bonatsou et al., 2018)
Candida	*Candida adriatica*	7.26	6.92	7.59	2	(Zullo and Ciafardini, 2019)
	Candida boidinii	12.82	3.53	22.1	2	(Porru et al., 2018)
		18.17	18.17	18.17	1	(Oliveira et al., 2017)
		60.47	0	81.6	6	(Bonatsou et al., 2018)
	Candida diddensiae	31.63	24.76	38.49	2	(Porru et al., 2018)
		0.45	0.28	0.62	2	(Zullo and Ciafardini, 2019)
		66.67	65.4	69	3	(Bonatsou et al., 2018)
	Candida molendinolei	62.63	51	89.8	7	(Bonatsou et al., 2018)
	Candida naeodendra	35.85	0	71.7	2	(Bonatsou et al., 2018)
	Candida norvegica	125.75	125.75	125.75	1	(Oliveira et al., 2017)
	Candida silvae	60.22	60.22	60.22	1	(S Bonatsou et al., 2015)
	Candida tropicalis	58.76	56.11	61.41	2	(Oliveira et al., 2017)
Citeromyces	*Citeromyces matrinensis*	55.3	55.3	55.3	1	(Bonatsou et al., 2018)
Cystofilobasidium	*Cystofilobasidium bisporidii*	87.5	87.5	87.5	1	(Bonatsou et al., 2018)
Debaryomyces	*Debaryomyces hansenii*	97.74	97.74	97.74	1	(Oliveira et al., 2017)
		0.08	0.08	0.08	1	(S Bonatsou et al., 2015)
Metschnikowia	*Metschnikowia pulcherrima*	12.1	12.1	12.1	1	(S Bonatsou et al., 2015)
		89.4	87.7	91.2	3	(Stamatoula Bonatsou et al., 2018)
Nakazawaea	*Nakazawaea molendini-olei*	0.02	0.02	0.02	1	(Porru et al., 2018)
		0.28	0.25	0.31	2	(Zullo and Ciafardini, 2019)
	Nakazawaea wickerhamii	2.34	2.34	2.34	1	(Zullo and Ciafardini, 2019)

pH 8.0, pancreatin 0.1 g/L, bile salts 3.0g/L, 3.5h, 37°C) Buffer:50.81 g/L of sodium phosphate dibasic heptahydrate and 8.5 g/L of NaCl

Appendix A.2: The survivability of selected yeast strains undergo in-vitro intestinal conditions (Continued)

Genus	Species	Survival	Minimum	Maximum	Count	Reference
Pichia	*Pichia guilliermondii*	68.91	68.91	68.91	1	(Oliveira et al., 2017)
		63.8	63.8	63.8	1	(Bonatsou et al., 2015)
		81.85	81.2	82.5	2	(Bonatsou et al., 2018)
	Pichia kluyveri	40.84	40.84	40.84	1	(Bonatsou et al., 2015)
		72.25	64.4	80.1	2	(Bonatsou et al., 2018)
		57.28	57.28	57.28	1	(Bonatsou et al., 2015)
		79.87	78.4	82.5	3	(Bonatsou et al., 2018)
	Pichia membranifaciens	11.29	11.29	11.29	1	(Bonatsou et al., 2015)
Rhodotorula	*Rhodotorula diobovatum*	3.83	3.83	3.83	1	(Bonatsou et al., 2015)
	Rhodotorula glutinis	70.6	62.4	76.8	3	(Bonatsou et al., 2018)
	Rhodotorula mucilaginosa	53.38	53.38	53.38	1	(Bonatsou et al., 2015)
		71.1	71.1	71.1	1	(Stamatoula Bonatsou et al., 2018)
Saccharomyces	*Saccharomyces boulardii*	10.99	10.99	10.99	1	(Porru et al., 2018)
		99.79	99.79	99.79	1	(Oliveira et al., 2017)
		6.92	6.92	6.92	1	(Zullo and Ciafardini, 2019)
	Saccharomyces cerevisiae	46.6	46.6	46.6	1	(Porru et al., 2018)
		94.29	12.61	175.98	2	(Oliveira et al., 2017)
		64.28	64.28	64.28	1	(Bonatsou et al., 2015)
		54.42	6.7	90.9	6	(Bonatsou et al., 2018)
Wickerhamomyces	*Wickerhamomyces anomalus*	5.28	5.28	5.28	1	(Porru et al., 2018)
		13.39	13.39	13.39	1	(Bonatsou et al., 2015)
		0.21	0.08	0.35	2	(Zullo and Ciafardini, 2019)
Yamadazyma	*Yamadazyma terventina*	1.93	1.41	2.45	2	(Zullo and Ciafardini, 2019)
Zygoascus	*Zygoascus hellenicus*	72.4	65.1	86.5	3	(Bonatsou et al., 2018)
Zygosaccharomyces	*Zygosaccharomyces mrakii*	0.1	0.1	0.1	1	(Porru et al., 2018)

pH 8.0, pancreatin 0.1 g/L, bile salts 3.0g/L, 3.5h, 37°C) Buffer:50.81 g/L of sodium phosphate dibasic heptahydrate and 8.5 g/L of NaCl

Appendix A.3: Evaluated of NIC (non-inhibitory concentration) and MIC (minimum inhibitory concentration) values (g/L) measured under salt (NaCl) for the yeast strains.

Genus	Species	NaCl g/L	pH	Time	Temp.	NIC (g/L) mean	Min.	Max.	MIC (g/L) mean	Min.	Maxi.	C.	Reference
Aureobasidium	*Aureobasidium pullulans*	0-250	3.5	12h for 7days	N.M	24.6	0	42.4	115.5	0	207.2	6	(Bonatsou et al., 2018)
			5	12h for 7days	N.M	49.98	41.8	71.2	162.97	110.6	191.5	6	(Bonatsou et al., 2018)
			6.5	12h for 7days	N.M	49.58	17.4	69.4	131.5	94.7	168.6	6	(Bonatsou et al., 2018)
		0-250	N.M	2h	N.M	44.47	44.5	44.5	127.09	127.1	127.1	1	(Bonatsou et al., 2015)
Candida	*Candida boidinii*	0-250	3.5	12h for 7days	N.M	52.85	26.8	74.9	103.8	89.8	117.3	6	(Bonatsou et al., 2018)
			5	12h for 7days	N.M	73.07	54.7	82.3	120.62	103.9	168.5	6	(Bonatsou et al., 2018)
			6.5	12h for 7days	N.M	70.22	58.8	82.1	113	88.6	130.2	6	(Bonatsou et al., 2018)
		0-180	4.5	7days	28	68.48	68.5	68.5	110.11	110.1	110.1	1	(Porru et al., 2018)
	Candida diddensiae	0-250	3.5	12h for 7days	N.M	48.77	0	92.4	81.37	0	155.2	3	(Bonatsou et al., 2018)
			5	12h for 7days	N.M	24.97	0	74.9	32.7	0	98.1	3	(Bonatsou et al., 2018)
			6.5	12h for 7days	N.M	19	0	57	29.53	0	88.6	3	(Bonatsou et al., 2018)

Media for all: YM broth; N.M: not mentioned; Min.: minimum; Max.: maximum.

Appendix A.3: Evaluated of NIC (non-inhibitory concentration) and MIC (minimum inhibitory concentration) values (g/L) measured under salt (NaCl) for the yeast strains (Continued).

Genus	Species	NaCl g/L	pH	Time	Temp.	NIC (g/L) mean	Min.	Max.	MIC (g/L) mean	Min.	Maxi.	C.	Reference
		0-180	4.5	7days	28	79.13	64.6	93.7	119.54	98.95	140.1	2	(Porru et al., 2018)
	Candida molendinolei	0-250	3.5	12h for 7days	N.M	47.11	38.8	52.7	127.57	123.4	140.9	7	(Bonatsou et al., 2018)
			5	12h for 7days	N.M	76.94	61.1	92.6	134.14	125.5	163.8	7	(Bonatsou et al., 2018)
			6.5	12h for 7days	N.M	59.31	38.3	98.1	128	113.3	154.7	7	(Bonatsou et al., 2018)
	Candida naeodendra	0-250	3.5	12h for 7days	N.M	49.7	24.3	75.1	512.35	117.7	907	2	(Bonatsou et al., 2018)
			5	12h for 7days	N.M	60.65	41.3	80	158.8	112.6	205	2	(Bonatsou et al., 2018)
			6.5	12h for 7days	N.M	81	76.6	85.4	111.85	101.3	122.4	2	(Stamato ula Bonatsou et al., 2018)
	Candida silvae	0-250	N.M	2h	N.M	45.82	45.8	45.8	134.66	134.7	134.7	1	(Bonatsou et al., 2015)
	Saccharomyces boidinii	0-180	4.5	7days	28	80.94	80.9	80.9	115.43	115.4	115.4	1	(Porru et al., 2018)
Citeromyces	*Citeromyces matrinensis*	0-250	3.5	12h for 7days	N.M	43.4	43.4	43.4	129.8	129.8	129.8	1	(Bonatsou et al., 2018)
			5	12h for 7days	N.M	0	0	0	0	0	0	1	(Bonatsou et al., 2018)
			6.5	12h for 7days	N.M	0	0	0	0	0	0	1	(Bonatsou et al., 2018)

Media for all: YM broth; N.M: not mentioned; Min.: minimum; Max.: maximum.

Appendix A.3: Evaluated of NIC (non-inhibitory concentration) and MIC (minimum inhibitory concentration) values (g/L) measured under salt (NaCl) for the yeast strains (Continued).

Genus	Species	NaCl g/L	pH	Time	Temp.	NIC (g/L) mean	Min.	Max.	MIC (g/L) mean	Min.	Maxi.	C.	Reference
Cystofilobasidium	*Cystofilobasidium bisporidii*	0-250	3.5	12h for 7days	N.M	17	17	17	88.8	88.8	88.8	1	(Bonatsou et al., 2018)
			5	12h for 7days	N.M	7.1	7.1	7.1	165.9	165.9	165.9	1	(Bonatsou et al., 2018)
			6.5	12h for 7days	N.M	4.8	4.8	4.8	205.3	205.3	205.3	1	(Bonatsou et al., 2018)
Debaryomyces	*Debaryomyces hansenii*	0-250	N.M	2h	N.M	50.22	50.2	50.2	153.59	153.6	153.6	1	(Bonatsou et al., 2015)
Metschnikowia	*Metschnikowia pulcherrima*	0-250	3.5	12h for 7days	N.M	22.73	0	43.7	66.47	0	130.3	3	(Stamato ula Bonatsou et al., 2018)
			5	12h for 7days	N.M	40.2	26.4	58.1	142.3	98.4	195.7	3	(Bonatsou et al., 2018)
			6.5	12h for 7days	N.M	20.7	0	34.2	76.43	0	133.4	3	(Bonatsou et al., 2018)
		0-250	N.M	2h	N.M	61.08	61.1	61.1	176.65	176.7	176.7	1	(Bonatsou et al., 2015)
Nakazawaea	*Nakazawaea molendiniolei*	0-180	4.5	7days	28	67.2	67.2	67.2	118.92	118.9	118.9	1	(Porru et al., 2018)
Pichia	*Pichia guilliermondii*	0-250	3.5	12h for 7days	N.M	24.2	0	48.4	82.85	0	165.7	2	(Bonatsou et al., 2018)
			5	12h for 7days	N.M	81.55	75.8	87.3	207.5	204.8	210.2	2	(Bonatsou et al., 2018)
			6.5	12h for 7days	N.M	62.45	61.7	63.2	226.85	174.8	278.9	2	(Bonatsou et al., 2018)
		0-250	N.M	2h	N.M	109.93	110	110	261.6	261.6	261.6	1	(Bonatsou et al., 2015)

Media for all: YM broth; N.M: not mentioned; Min.: minimum; Max.: maximum.

Appendix A.3: Evaluated of NIC (non-inhibitory concentration) and MIC (minimum inhibitory concentration) values (g/L) measured under salt (NaCl) for the yeast strains (Continued).

Genus	Species	NaCl g/L	pH	Time	Temp.	NIC (g/L) mean	Min.	Max.	MIC (g/L) mean	Min.	Maxi.	C.	Reference
	Pichia kluyveri	0-250	3.5	12h for 7days	N.M	86.5	70.9	102	156.05	149.4	162.7	2	(Bonatsou et al., 2018)
			5	12h for 7days	N.M	0	0	0	0	0	0	2	(Bonatsou et al., 2018)
			6.5	12h for 7days	N.M	0	0	0	0	0	0	2	(Bonatsou et al., 2018)
		0-250	N.M	2h	N.M	84.69	84.7	84.7	209.66	209.7	209.7	1	(Bonatsou et al., 2015)
	Pichia manshurica	0-250	3.5	12h for 7days	N.M	48.5	39.9	62	159.57	149.9	178.3	3	(Stamato ula Bonatsou et al., 2018)
			5	12h for 7days	N.M	60.67	50.2	70.5	134.37	128.4	144.5	3	(Bonatsou et al., 2018)
			6.5	12h for 7days	N.M	70	64	80.6	125.93	115.2	143.3	3	(Bonatsou et al., 2018)
		0-250	N.M	2h	N.M	71.16	71.2	71.2	152.13	152.1	152.1	1	(S Bonatsou et al., 2015)
	Pichia membranifaciens	0-250	N.M	2h	N.M	20.15	20.2	20.2	154.41	154.4	154.4	1	(Bonatsou et al., 2015)
Rhodotorula	*Rhodotorula diobovatum*	0-250	N.M	2h	N.M	49.97	50	50	123.7	123.7	123.7	1	(Bonatsou et al., 2015)
	Rhodotorula glutinis	0-250	3.5	12h for 7days	N.M	27.1	16.9	35.9	99.33	80.2	116.7	3	(Bonatsou et al., 2018)
			5	12h for 7days	N.M	26.73	14.3	41.3	131.8	74.8	183.4	3	(Bonatsou et al., 2018)
			6.5	12h for 7days	N.M	30.7	11.7	49.9	211.47	99.6	404.2	3	(Bonatsou et al., 2018)

Media for all: YM broth; N.M: not mentioned; Min.: minimum; Max.: maximum.

Appendix A.3: Evaluated of NIC (non-inhibitory concentration) and MIC (minimum inhibitory concentration) values (g/L) measured under salt (NaCl) for the yeast strains (Continued).

Appendix A.3: Evaluated of NIC (non-inhibitory concentration) and MIC (minimum inhibitory concentration) values (g/L) measured under salt (NaCl) for the yeast strains (Continued).

Genus	Species	NaCl g/L	pH	Time	Temp.	NIC (g/L) mean	Min.	Max.	MIC (g/L) mean	Min.	Maxi.	C.	Reference
	Rhodotorula mucilaginosa	0-250	3.5	12h for 7days	N.M	52.4	52.4	52.4	95.6	95.6	95.6	1	(Bonatsou et al., 2018)
			5	12h for 7days	N.M	52.2	52.2	52.2	122.6	122.6	122.6	1	(Bonatsou et al., 2018)
			6.5	12h for 7days	N.M	62.5	62.5	62.5	119.1	119.1	119.1	1	(Bonatsou et al., 2018)
		0-250	N.M	2h	N.M	58.26	58.3	58.3	266.02	266	266	1	(Bonatsou et al., 2015)
Saccharomyces	*Saccharomyces boulardii*	0-180	4.5	7days	28	24.54	24.5	24.5	86.39	86.39	86.39	1	(Porru et al., 2018)
	Saccharomyces cerevisiae	0-250	3.5	12h for 7days	N.M	35.5	21.8	45.9	146.73	119	200	6	(Bonatsou et al., 2018)
			5	12h for 7days	N.M	60.37	41.1	85.3	174.83	139.1	195.2	6	(Bonatsou et al., 2018)
			6.5	12h for 7days	N.M	40.03	0	67.1	143.8	0	214.9	6	(Bonatsou et al., 2018)
		0-180	4.5	7days	28	24.82	24.8	24.8	86.29	86.29	86.29	1	(Porru et al., 2018)
		0-250	N.M	2h	N.M	86.3	86.3	86.3	274.44	274.4	274.4	1	(Bonatsou et al., 2015)
Wickerhamomyces	*Wickerhamomyces anomalus*	0-180	4.5	7days	28	114.8	115	115	171.47	171.5	171.5	1	(Porru et al., 2018)
		0-250	N.M	2h	N.M	47.27	47.3	47.3	163.18	163.2	163.2	1	(Bonatsou et al., 2015)

Media for all: YM broth; N.M: not mentioned; Min.: minimum; Max.: maximum

Appendix A.3: Evaluated of NIC (non-inhibitory concentration) and MIC (minimum inhibitory concentration) values (g/L) measured under salt (NaCl) for the yeast strains (Continued).

Genus	Species	NaCl g/L	pH	Time	Temp.	NIC (g/L) mean	Min.	Max.	MIC (g/L) mean	Min.	Maxi.	C.	Reference
Zygoascus	*Zygoascus hellenicus*	0-250	3.5	12h for 7days	N.M	76.53	37.7	96.4	125.23	115.4	143.2	3	(Bonatsou et al., 2018)
			5	12h for 7days	N.M	81.23	68.8	93.7	147.13	146.6	147.9	3	(Bonatsou et al., 2018)
			6.5	12h for 7days	N.M	54.43	28.3	78.3	129.63	112.9	157.9	3	(Bonatsou et al., 2018)
Zygosaccharomyces	*Zygosaccharomyces mrakii*	0-180	4.5	7days	28	40.55	40.6	40.6	96.74	96.74	96.74	1	(Porru et al., 2018)

Media for all: YM broth; N.M: not mentioned; Min.: minimum; Max.: maximum

Appendix A.4: The percentage of auto-aggregation capacity of the selected yeast strains, incubated at 37°C and measured at different time intervals.

Genus	Species	Time	Media	Mean	Au. Min.	Au. Max.	C.	Reference
Aureobasidium	*Aureobasidium pullulans*	24h	PBS	91.25	84	95	4	(Bonatsou et al., 2018)
		2h	PBS	84.75	76	94	4	(Bonatsou et al., 2018)
		4h	PBS	84	80	88	4	(Bonatsou et al., 2018)
Candida	*Candida adriatica*	4h	Sodium Phosphate buffer	21	10	32	2	(Zullo and Ciafardini, 2019)
	Candida boidinii	24h	PBS	93.5	90	96	4	(Bonatsou et al., 2018)
		2h	PBS	77	58	90	4	(Bonatsou et al., 2018)
		4h	PBS	74.25	57	84	4	(Bonatsou et al., 2018)
		24h	0.9%NaCl salin solution	50.97	25.36	76.58	2	(Oliveira et al., 2017)
		2h	0.9%NaCl salin solution	17.48	8.96	26	2	(Oliveira et al., 2017)
		4h	0.9%NaCl salin solution	28	14	42	2	(Oliveira et al., 2017)
	Candida Caribbica	24h	PBS	99	99	99	2	(Amorim et al., 2018)
	Candida diddensiae	24h	PBS	92	92	92	2	(Bonatsou et al., 2018)
		2h	PBS	60	60	60	2	(Bonatsou et al., 2018)
		4h	PBS	64	64	64	2	(Bonatsou et al., 2018)
		4h	Sodium Phosphate buffer	35.5	23	48	2	(Zullo and Ciafardini, 2019)
	Candida Lusitaniae	24h	PBS	96	96	96	3	(Amorim et al., 2018)
		2h	PBS	4	0	7	3	(Amorim et al., 2018)
		4h	PBS	40.67	38	43	3	(Amorim et al., 2018)
	Candida molendinolei	24h	PBS	92.67	90	96	3	(Bonatsou et al., 2018)
		2h	PBS	42.33	22	75	3	(Bonatsou et al., 2018)
		4h	PBS	48.33	30	78	3	(Bonatsou et al., 2018)
	Candida naeodendra	24h	PBS	94	94	94	1	(Bonatsou et al., 2018)
		2h	PBS	83	83	83	1	(Bonatsou et al., 2018)

Aut. Min: autoaggregation minimum; Aut.Max.: autoaggregation maximum.

Appendix A.4: The percentage of auto-aggregation capacity of the selected yeast strains, incubated at 37°C and measured at different time intervals (Continued).

Genus	Species	Time	Media	Mean	Au. Min.	Au. Max.	C.	Reference
		4h	PBS	79	79	79	1	(Bonatsou et al., 2018)
	Candida norvegica	24h	0.9%NaCl salin solution	83	83	83	1	(Oliveira et al., 2017)
		2h	0.9%NaCl salin solution	38.27	38.27	38.27	1	(Oliveira et al., 2017)
		4h	0.9%NaCl salin solution	59	59	59	1	(Oliveira et al., 2017)
	Candida orthopsilosis	5h	PBS	87.5	87	88	2	(Menezes et al., 2020)
	Candida pararugosa	30min	0.9%NaCl salin solution	63	63	63	1	(Fernandez-Pacheco Rodríguez et al., 2018)
	Candida quercitrusa	5h	PBS	96	96	96	1	(Menezes et al., 2020)
	Candida sake	30min	0.9%NaCl salin solution	16	16	16	1	(Fernandez-Pacheco Rodríguez et al., 2018)
	Candida tropicalis	24h	0.9%NaCl salin solution	86.16	78.37	93.94	2	(Oliveira et al., 2017)
		2h	0.9%NaCl salin solution	70.5	66	75	2	(Oliveira et al., 2017)
		4h	0.9%NaCl salin solution	74.42	71.29	77.54	2	(Oliveira et al., 2017)
	Candida vini	30min	0.9%NaCl salin solution	42	42	42	1	(Fernandez-Pacheco Rodríguez et al., 2018)
Cystofilobasidium	*Cystofilobasidium bisporidii*	24h	PBS	90	90	90	1	(Bonatsou et al., 2018)
		2h	PBS	37	37	37	1	(Bonatsou et al., 2018)
		4h	PBS	36	36	36	1	(Bonatsou et al., 2018)
Debaryomyce	*Debaryomyces hansenii*	24h	0.9%NaCl salin solution	34.37	34.37	34.37	1	(Oliveira et al., 2017)
		2h	0.9%NaCl salin solution	31	31	31	1	(Oliveira et al., 2017)
		4h	0.9%NaCl salin solution	30.83	30.83	30.83	1	(Oliveira et al., 2017)
Galactomyces	*Galactomyces reessii*	24h	0.9%NaCl salin solution	45.33	31	70.5	3	(Oliveira et al., 2017)
		2h	0.9%NaCl salin solution	28.44	22.01	34.86	2	(Oliveira et al., 2017)
		4h	0.9%NaCl salin solution	29.21	29.21	29.21	1	(Oliveira et al., 2017)

Aut. Min: autoaggregation minimum; Aut.Max.: autoaggregation maximum.

Appendix A.4: The percentage of auto-aggregation capacity of the selected yeast strains, incubated at 37°C and measured at different time intervals (Continued).

Genus	Species	Time	Media	Mean	Au. Min.	Au. Max.	C.	Reference
Hanseniaspora	*Hanseniaspora opuntiae*	5h	PBS	66	66	66	1	(Menezes et al., 2020)
	Hanseniaspora osmophila	30min	0.9%NaCl salin solution	48	32	64	2	(Fernandez-Pacheco Rodríguez et al., 2018)
	Hanseniaspora uvarum	5h	PBS	85	84	86	2	(Menezes et al., 2020)
Kluyveromyces	*Kluyveromyces lactis*	2h	PBS	35	35	35	1	(Gut et al., 2019)
	Kluyveromyces marxianus	5h	PBS	83	76	90	2	(Menezes et al., 2020)
	Kluyveromyces thermotolerans	30min	0.9%NaCl salin solution	28.5	14	43	2	(Fernandez-Pacheco Rodríguez et al., 2018)
Metschnikowia	*Metschnikowia pulcherrima*	24h	PBS	90	85	94	3	(Bonatsou et al., 2018)
		2h	PBS	62.33	28	80	3	(Bonatsou et al., 2018)
		4h	PBS	66	31	84	3	(Bonatsou et al., 2018)
		30min	0.9%NaCl salin solution	13	13	13	1	(Fernandez-Pacheco Rodríguez et al., 2018)
Meyerozyma	*Meyerozyma Caribbica*	2h	PBS	14.5	14	15	2	(Amorim et al., 2018)
		4h	PBS	40.5	36	45	2	(Amorim et al., 2018)
Nakazawaea	*Nakazawaea molendini-olei*	4h	Sodium Phosphate buffer	18	8	28	2	(Zullo and Ciafardini, 2019)
	Nakazawaea wickerhamii	4h	Sodium Phosphate buffer	18	18	18	1	(Zullo and Ciafardini, 2019)

Aut. Min: autoaggregation minimum; Aut.Max.: autoaggregation maximum.

Appendix A.4: The percentage of auto-aggregation capacity of the selected yeast strains, incubated at 37°C and measured at different time intervals (Continued).

Genus	Species	Time	Media	Mean	Au. Min.	Au. Max.	C.	Reference
Ogataea	*Ogataea polymorpha*	30min	0.9%NaCl salin solution	4	4	4	1	(Fernandez-Pacheco Rodríguez et al., 2018)
Pichia	*Pichia membranifaciens*	24h	0.9%NaCl salin solution	76.6	76.13	77.08	2	(Oliveira et al., 2017)
	Pichia anomala	30min	0.9%NaCl salin solution	19.5	11	28	2	(Fernandez-Pacheco Rodríguez et al., 2018)
	Pichia caribbica	30min	0.9%NaCl salin solution	7	7	7	1	(Fernandez-Pacheco Rodríguez et al., 2018)
	Pichia galeiforms	30min	0.9%NaCl salin solution	12	12	12	1	(Fernandez-Pacheco Rodríguez et al., 2018)
	Pichia guilliermondii	5h	PBS	83	83	83	1	(Menezes et al., 2020)
		24h	0.9%NaCl salin solution	73.66	73.66	73.66	1	(Oliveira et al., 2017)
		2h	0.9%NaCl salin solution	25	25	25	1	(Oliveira et al., 2017)
		4h	0.9%NaCl salin solution	30.66	30.66	30.66	1	(Oliveira et al., 2017)
	Pichia kluyveri	24h	PBS	90	88	92	2	(Bonatsou et al., 2018)
		2h	PBS	45	41	49	2	(Bonatsou et al., 2018)
		4h	PBS	53	48	58	2	(Bonatsou et al., 2018)
		5h	PBS	92	92	92	1	(Menezes et al., 2020)
		30min	0.9%NaCl salin solution	30	30	30	1	(Fernandez-Pacheco Rodríguez et al., 2018)
	Pichia kudriavzevii	30min	0.9%NaCl salin solution	16.5	5	28	2	(Fernandez-Pacheco Rodríguez et al., 2018)

Aut. Min: autoaggregation minimum; Aut.Max.: autoaggregation maximum.

Appendix A.4: The percentage of auto-aggregation capacity of the selected yeast strains, incubated at 37°C and measured at different time intervals (Continued).

Genus	Species	Time	Media	Mean	Au. Min.	Au. Max.	C.	Reference
	Pichia manshurica	24h	PBS	91	89	92	3	(Bonatsou et al., 2018)
		2h	PBS	77	61	88	3	(Bonatsou et al., 2018)
		4h	PBS	75	55	89	3	(Bonatsou et al., 2018)
		24h	0.9%NaCl salin solution	76.86	76.86	76.86	1	(Oliveira et al., 2017)
		2h	0.9%NaCl salin solution	13	13	13	1	(Oliveira et al., 2017)
		4h	0.9%NaCl salin solution	24.75	24.75	24.75	1	(Oliveira et al., 2017)
	Pichia membranifaciens	5h	PBS	93	93	93	1	(Menezes et al., 2020)
		2h	0.9%NaCl salin solution	51.58	48.16	55	2	(Oliveira et al., 2017)
		4h	0.9%NaCl salin solution	56.5	47	66	2	(Oliveira et al., 2017)
		30min	0.9%NaCl salin solution	41	41	41	1	(Fernandez-Pacheco Rodríguez et al., 2018)
Rhodotorula	*Rhodotorula glutinis*	24h	PBS	91.67	87	96	3	(Bonatsou et al., 2018)
		2h	PBS	50	38	62	3	(Bonatsou et al., 2018)
		4h	PBS	66	46	80	3	(Bonatsou et al., 2018)
		24h	0.9%NaCl salin solution	23.67	23.67	23.67	1	(Oliveira et al., 2017)
		2h	0.9%NaCl salin solution	2.86	2.86	2.86	1	(Oliveira et al., 2017)
		4h	0.9%NaCl salin solution	5.12	5.12	5.12	1	(Oliveira et al., 2017)
	Rhodotorula graminis	24h	0.9%NaCl salin solution	20.11	20.11	20.11	1	(Oliveira et al., 2017)
		2h	0.9%NaCl salin solution	3.35	3.35	3.35	1	(Oliveira et al., 2017)
		4h	0.9%NaCl salin solution	4	4	4	1	(Oliveira et al., 2017)
	Rhodotorula mucilaginosa	24h	PBS	90	90	90	1	(Bonatsou et al., 2018)
		2h	PBS	74	74	74	1	(Bonatsou et al., 2018)
		4h	PBS	74	74	74	1	(Bonatsou et al., 2018)

Aut. Min: autoaggregation minimum; Aut.Max.: autoaggregation maximum.

Appendix A.4: The percentage of auto-aggregation capacity of the selected yeast strains, incubated at 37°C and measured at different time intervals (Continued).

Genus	Species	Time	Media	Mean	Au. Min.	Au. Max.	C.	Reference
Saccharomyces	*Saccharomyces boulardii*	5h	PBS	96	96	96	1	(Menezes et al., 2020)
		24h	0.9%NaCl salin solution	55.9	34.75	77.05	2	(Oliveira et al., 2017)
		2h	0.9%NaCl salin solution	25.5	25.5	25.5	1	(Oliveira et al., 2017)
		4h	Sodium Phosphate buffer	43	43	43	1	(Zullo and Ciafardini, 2019)
		2h	PBS	37	31	43	2	(Gut et al., 2019)
	Saccharomyces cerevisiae	24h	PBS	95.25	94	96	4	(Bonatsou et al., 2018)
		2h	PBS	84.75	72	90	4	(Bonatsou et al., 2018)
		4h	PBS	82.5	67	91	4	(Bonatsou et al., 2018)
		5h	PBS	94.07	68	99	14	(Menezes et al., 2020)
		24h	0.9%NaCl salin solution	83.02	74.33	91.71	2	(Oliveira et al., 2017)
		2h	0.9%NaCl salin solution	56.99	54	59.97	2	(Oliveira et al., 2017)
		4h	0.9%NaCl salin solution	71.62	71.23	72	2	(Oliveira et al., 2017)
		30min	0.9%NaCl salin solution	20	20	20	1	(Fernandez-Pacheco Rodríguez et al., 2018)
	Saccharomyces cerevisiae var. boulardii	30min	0.9%NaCl salin solution	19	19	19	1	(Fernandez-Pacheco Rodríguez et al., 2018)
		24h	PBS	96	96	96	1	(Amorim et al., 2018)
		2h	PBS	3	3	3	1	(Amorim et al., 2018)
		4h	PBS	44	44	44	1	(Amorim et al., 2018)
	Saccharomyces unisporus	2h	PBS	43	43	43	1	(Gut et al., 2019)
	Saccharomycescerevisiae	5h	PBS	96	96	96	1	(Menezes et al., 2020)
Torulaspora	*Torulaspora delbrueckii*	30min	0.9%NaCl salin solution	19	19	19	1	(Fernandez-Pacheco Rodríguez et al., 2018)
Wickerhamomyces	*Wickerhamomyces anomalus*	4h	Sodium Phosphate buffer	30	28	32	2	(Zullo and Ciafardini, 2019)

Aut. Min: autoaggregation minimum; Aut.Max.: autoaggregation maximum.

Appendix A.4: The percentage of auto-aggregation capacity of the selected yeast strains, incubated at 37°C and measured at different time intervals (Continued).

Genus	Species	Time	Media	Mean	Au. Min.	Au. Max.	C.	Reference
Yamadazyma	*Yamadazyma terventina*	4h	Sodium Phosphate buffer	31.5	30	33	2	(Zullo and Ciafardini, 2019)
Zygoascus	*Zygoascus hellenicus*	24h	PBS	90.67	88	92	3	(Bonatsou et al., 2018)
		2h	PBS	43	25	65	3	(Bonatsou et al., 2018)
		4h	PBS	51	36	70	3	(Bonatsou et al., 2018)
Zygosaccharomyces	*Zygosaccharomyces bailii*	30min	0.9%NaCl salin solution	35	35	35	1	(Fernandez-Pacheco Rodríguez et al., 2018)
	Zygosaccharomyces fermentati	30min	0.9%NaCl salin solution	13	4	26	3	(Fernandez-Pacheco Rodríguez et al., 2018)

Aut. Min: autoaggregation minimum; Aut.Max.: autoaggregation maximum.

Appendix A.5: The percentage of hydrophobicity capacity of the selected yeast strains, incubated at 37°C and measured at different time intervals.

						Hexadecane		Xylene		Toluene	
Genus	**Species**	**Time**	**Temp.°C**	**Media**	**Reference**	**Mean**	**C.**	**Mean**	**C.**	**Mean**	**C.**
Candida	*Candida adriatica*	1h	N.M	PBS	(Zullo and Ciafardini, 2019)	37.5	2	.	2	.	2
	Candida diddensiae	1h	N.M	PBS	(Zullo and Ciafardini, 2019)	29	2	.	2	.	2
	Candida Lusitaniae	30 min	37	PBS	(Amorim et al., 2018)	91.9	1	99. 66	1	.	1
	Candida orthopsilosis	1h	37	Phosphate Buffer	(Menezes et al., 2020)	98.9	2	.	2	.	2
	Candida pararugosa	1h	37	KNO3 solution	(Fernandez-Pacheco Rodríguez et al., 2018)	51.9	1	.	1	25. 87	1
	Candida quercitrusa	1h	37	Phosphate Buffer	(Menezes et al., 2020)	98.9	1	.	1	.	1
	Candida sake	1h	37	KNO3 solution	(Fernandez-Pacheco Rodríguez et al., 2018)	6.64	1	.	1	13.35	1
	Candida vini	1h	37	KNO3 solution	(Fernandez-Pacheco Rodríguez et al., 2018)	30.81	1	.	1	33.09	1
Hanseniaspora	*Hanseniaspora opuntiae*	1h	37	Phosphate Buffer	(Menezes et al., 2020)	98.7	1	.	1	.	1
	Hanseniaspora osmophila	1h	37	KNO3 solution	(Fernandez-Pacheco Rodríguez et al., 2018)	32.2	2	.	2	30.22	2
	Hanseniaspora uvarum	1h	37	Phosphate Buffer PBS	(Menezes et al., 2020)	98	2	.	2	.	2
Kluyveromyces	*Kluyveromyces lactis*	1h	37	Buffer PBS	(Gut et al., 2019)	88.75	1	.	1	.	1
	Kluyveromyces marxianus	1h	37	Phosphate Buffer	(Menezes et al., 2020)	99.45	2	.	2	.	2
	Kluyveromyces thermotolerans	1h	37	KNO3 solution	(Fernandez-Pacheco Rodrgí uez et al., 2018)	20.77	2	.	2	14. 46	2
Metschnikowia	*Metschnikowia pulcherrima*	1h	37	KNO3 solution	(Fernandez-Pacheco Rodrgí uez et al., 2018)	15.22	1	.	1	15.05	1

C.: count

Appendix A.5: The percentage of hydrophobicity capacity of the selected yeast strains, incubated at 37°C and measured at different time intervals (Continued).

						Hexadecane		Xylene		Toluene	
Genus	**Species**	**Time**	**Temp.°C**	**Media**	**Reference**	**Mean**	**C.**	**Mean**	**C.**	**Mean**	**C.**
Meyerozyma	*Meyerozyma Caribbica*	30 min	37	PBS	(Amorim et al., 2018)	68.2	2	64.13	2	.	2
Nakazawaea	*Nakazawaea molendiniolei*	1h	N.M	PBS	(Zullo and Ciafardini, 2019)	0	2	.	2	.	2
	Nakazawaea wickerhamii	1h	N.M	PBS	(Zullo and Ciafardini, 2019)	0	1	.	1	.	1
Ogataea	*Ogataea polymorpha*	1h	37	KNO3 solution	(Fernandez-Pacheco Rodríguez et al., 2018) uez et al., 2018)	10.55	1	.	1	5.44	1
Pichia	*Pichia anomala*	1h	37	KNO3 solution	(Fernandez-Pacheco Rodríguez et al., 2018)	27.16	2	.	2	31. 71	2
	Pichia caribbica	1h	37	KNO3 solution	(Fernandez-Pacheco Rodríguez et al., 2018)	3.1.6	1	. .	1	10. 76	1
	Pichia galeiforms	1h	37	KNO3 solution	(Fernandez-Pacheco Rodríguez et al., 2018)	21.49	1	.	1	29.6	1
	Pichia guilliermondii	1h	37	Phosphate Buffer	(Menezes et al., 2020)	94.9	1	.	1	.	1
	Pichia kluyveri	1h	37	Phosphate Buffer	(Menezes et al., 2020)	99.3	1	.	1	.	1
	Pichia kluyveri	1h	37	KNO3 solution	(Fernandez-Pacheco Rodríguez et al., 2018)	37.9	1	.	1	10.55	1
	Pichia kudriavzevii	1h	37	KNO3 solution	(Fernandez-Pacheco Rodríguez et al., 2018)	27.54	2	.	2	11.13	2
	Pichia membranifaciens	1h	37	Phosphate Buffer	(Menezes et al., 2020)	99.7	1	.	1		1
	Pichia membranifaciens	1h	37	KNO3 solution	(Fernandez-Pacheco Rodríguez et al., 2018)	33.4	1	.	1	15.66	1
	Saccharomyces boulardii	1h	37	PBS	(Gut et al., 2019)	18.38	2	.	2	.	2

C.: count

Appendix A.5: The percentage of hydrophobicity capacity of the selected yeast strains, incubated at 37°C and measured at different time intervals (Continued).

						Hexadecane		Xylene		Toluene	
Genus	**Species**	**Time**	**Temp.°C**	**Media**	**Reference**	**Mean**	**C.**	**Mean**	**C.**	**Mean**	**C.**
Saccharomyces	*Saccharomyces boulardii*	1h	37	Phosphate Buffer	(Menezes et al., 2020)	99.4	1	.	1	.	1
	Saccharomyces boulardii	1h	N.M	PBS	(Zullo and Ciafardini, 2019)	26	1	.	1	.	1
	Saccharomyces cerevisiae	1h	37	Phosphate Buffer	(Menezes et al., 2020)	99.52	15	.	15	.	15
	Saccharomyces cerevisiae	1h	37	KNO3 solution	(Fernandez-Pacheco Rodríguez et al., 2018)	38.74	1	.	1	26.68	1
	Saccharomyces cerevisiae var.boulardii	1h	37	KNO3 solution	(Fernandez-Pacheco Rodríguez et al., 2018)	37.15	1	.	1	32.41	1
	Saccharomyces unisporus	1h	37	PBS	(Gut et al., 2019)	30	1	.	1	.	1
Torulasporaomyces	*Torulaspora delbrueckii*	1h	37	KNO3 solution	(Fernandez-Pacheco Rodríguez et al., 2018)	37.41	1	.	1	19.34	1
Wickerham	*Wickerhamomyces anomalus*	1h	N.M	PBS	(Zullo and Ciafardini, 2019)	22.25	2	.	2	.	2
Yamadazyma	*Yamadazyma terventina*	1h	N.M	PBS	(Zullo and Ciafardini, 2019)	41.5	2	.	2	.	2
Zygosaccharomyces	*Zygosaccharomyces bailii*	1h	37	KNO3 solution	(Fernandez-Pacheco Rodríguez et al., 2018)	54.16	1	.	1	35.11	1
	Zygosaccharomyces fermentati	1h	37	KNO3 solution	(Fernandez-Pacheco Rodríguez et al., 2018)	9.59	3	.	3	64.7	3

C.: count

Appendix B.1: Acid tolerance at pH 2.5 during 24 h of incubation at 37°C for 105 potential probiotic yeast isolates

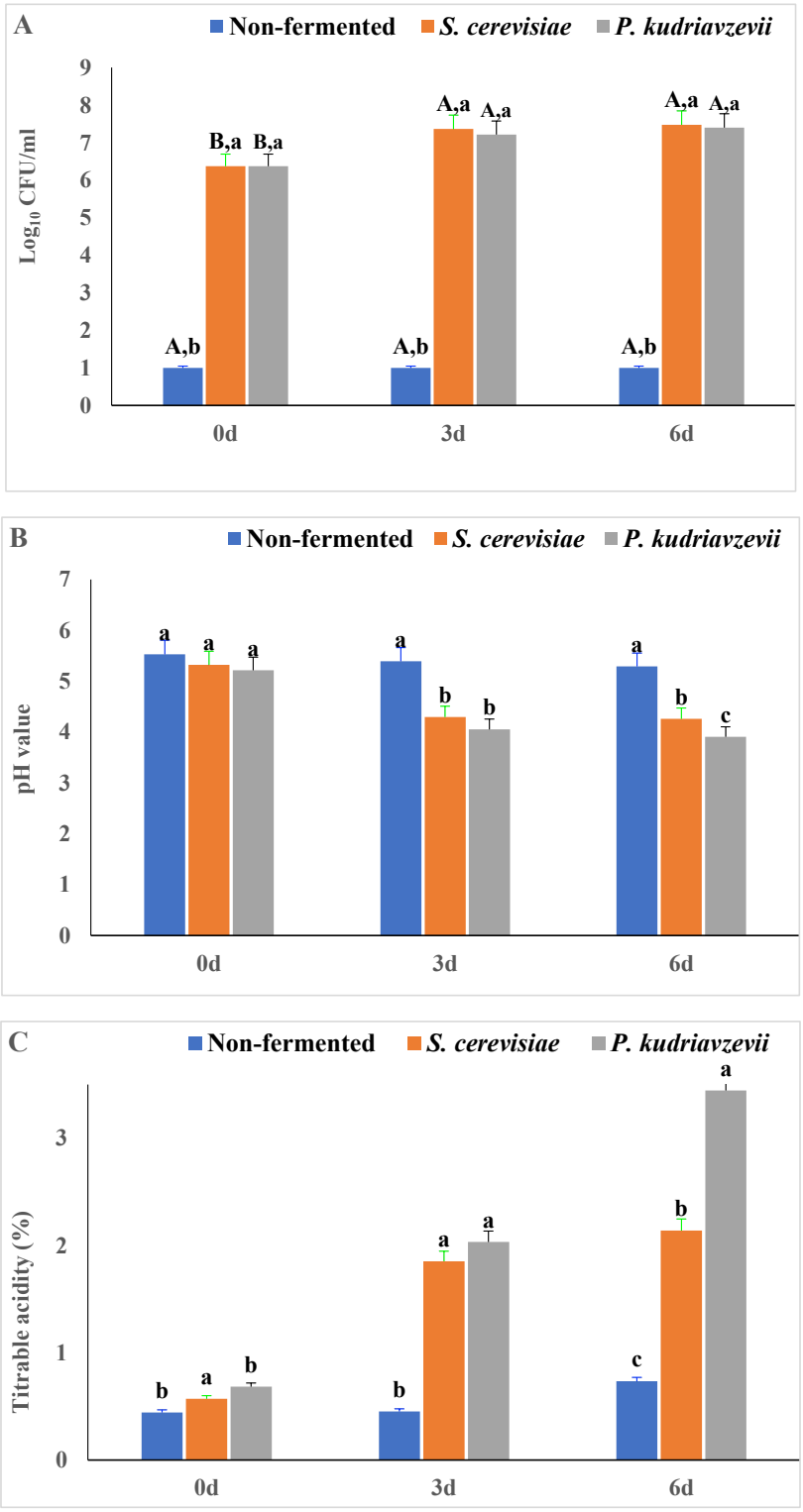
Isolate	Source	Survival rate (%)
G1	Gamed (dairy product)	76.1±3.8
G2	Gamed (dairy product)	74.1±3.7
G3	Gamed (dairy product)	74.9±3.7
G4	Gamed (dairy product)	69.4±3.4
G5	Gamed (dairy product)	57.4±2.8
G6	Gamed (dairy product)	85.1±4.2
G7	Gamed (dairy product)	75.1±3.7
G8	Gamed (dairy product)	111.2±5.5
G9	Gamed (dairy product)	79.1±3.9
G10	Gamed (dairy product)	83.1±4.1
O11	Egyptian Olive (fermented black olive pickle)	45.3±2.2
O12	Jardanian Olive (fermented black olive pickle)	105.8±5.2
O13	Jardanian Olive (fermented black olive pickle)	84.8±4.2
O14	Jardanian Olive (fermented black olive pickle)	70.4±3.5
O15	Jardanian Olive (fermented black olive pickle)	80.1±4.1
O16	Jardanian Olive (fermented black olive pickle)	76.1±3.8
O17	Jardanian Olive (fermented black olive pickle)	78.1±3.9
O18	Jardanian Olive in oil (fermented black olive pickle)	95.3±4.7
O19	Jardanian Olive in oil (fermented black olive pickle)	100.1±5
O20	Jardanian Olive in oil (fermented black olive pickle)	94.2±4.7
O21	Jardanian Olive in oil (fermented black olive pickle)	100.6±5
O22	Jardanian Olive in oil (fermented black olive pickle)	92.1±4.6
O23	Jardanian Olive in oil (fermented black olive pickle)	86.7±4.3
O24	Jardanian Olive in oil (fermented black olive pickle)	90.3±4.5
O25	Moroccan green olives (fermented black olive pickle)	83.8±4.1
O26	Moroccan green olives (fermented green olive pickle)	86.1±4.3
O27	Moroccan green olives (fermented green olive pickle)	100.1±5
O28	Jordanian green olives (fermented green olive pickle)	102.6±5.1
O29	Jordanian green olives (fermented green olive pickle)	119.3±5.9
O30	Jordanian green olives (fermented green olive pickle)	83.8±4.1
O31	Jordanian green olives (fermented green olive pickle)	70.3±3.5
O32	Jordanian green olives (fermented green olive pickle)	95.1±4.7
O33	Jordanian green olives (fermented green olive pickle)	106.5±5.3
O34	Jordanian green olives (fermented green olive pickle)	82.6±4.1
O35	Jordanian green olives (fermented green olive pickle)	105.1±5.2

Appendix B.1: Acid tolerance at pH 2.5 during 24 h of incubation at 37°C for 105 potential probiotic yeast isolates (Continued).

Isolate	Source	Survival rate (%)
O36	Jordanian green olives (fermented green olive pickle)	85.1±4.2
CH37	Brie cheese (soft cow's-milk cheese)	85.7±4.2
SH38	Shanklish (dried Labanah traditional fermented by fungi)	57.1±2.8
SH39	Shanklish (dried Labanah traditional fermented by fungi)	82.9±4.1
SH40	Shanklish (dried Labanah traditional fermented by fungi)	94.8±4.7
SH41	Shanklish (dried Labanah traditional fermented by fungi)	68.1±3.4
SH42	Shanklish (dried Labanah traditional fermented by fungi)	85.1±4.2
SH43	Shanklish (dried Labanah traditional fermented by fungi)	47.8±2.3
SH44	Shanklish (dried Labanah traditional fermented by fungi)	20.9±1.0
SH45	Shanklish (dried Labanah traditional fermented by fungi)	96.1±4.8
SH46	Shanklish (dried Labanah traditional fermented by fungi)	92.2±4.6
SH47	Shanklish (dried Labanah traditional fermented by fungi)	80.9±4.0
SH48	Shanklish (dried Labanah traditional fermented by fungi)	68.8±3.4
SH49	Shanklish (dried Labanah traditional fermented by fungi)	86.5±4.3
SH50	Shanklish (dried Labanah traditional fermented by fungi)	87.1±4.3
SH51	Shanklish (dried Labanah traditional fermented by fungi)	75.1±3.7
SH52	Shanklish (dried Labanah traditional fermented by fungi)	71.4±3.5
SH53	Shanklish (dried Labanah traditional fermented by fungi)	73.3±3.6
SH54	Shanklish (dried Labanah traditional fermented by fungi)	133.5±6.6
SH55	Shanklish (dried Labanah traditional fermented by fungi)	86.9±4.3
SH56	Shanklish (dried Labanah traditional fermented by fungi)	63.9±3.1
D57	Date fruit	44.6±2.2
D58	Date fruit	34.3±1.7
D59	Date fruit	50.2±2.5
O60	Egyptian Olive (fermented black olive pickle)	87.1±4.3
O61	Egyptian Olive (fermented black olive pickle)	96.4±4.8
O62	Jordan green olives (fermented black olive pickle)	81.6±4.0
O63	Jordanian green olives (fermented black olive pickle)	115.5±5.7
O64	Jordanian green olives (fermented black olive pickle)	96.7±4.8
O65	Jordanian green olives (fermented black olive pickle)	92.1±4.6
O66	Jordanian green olives (fermented black olive pickle)	91.7±4.5
O67	Jordanian green olives (fermented black olive pickle)	40.2±2.0
O68	Jordanian green olives (fermented black olive pickle)	44.9±2.2
O69	Gamed (dairy product)	75.4±3.7
G70	Gamed (dairy product)	59.6±2.9
G71	Gamed (dairy product)	79.2±3.9
G72	Gamed (dairy product)	51.1±2.5
G73	Gamed (dairy product)	70.8±3.5
G74	Gamed (dairy product)	37.5±1.8

Appendix B.1: Acid tolerance at pH 2.5 during 24 h of incubation at 37°C for 105 potential probiotic yeast isolates (Continued).

Isolate	Source	Survival rate (%)
G75	Gamed (dairy product)	79.2±3.9
G76	Gamed (dairy product)	19.4±0.9
G77	Gamed (dairy product)	88.6±4.4
G78	Gamed (dairy product)	78.3±3.9
G79	Gamed (dairy product)	51.5±2.5
G80	Gamed (dairy product)	75.7±3.7
G81	Gamed (dairy product)	50.2±2.5
G82	Gamed (dairy product)	74.1±3.7
G83	Gamed (dairy product)	36.1±1.8
G84	Gamed (dairy product)	77.2±3.8
G85	Gamed (dairy product)	101.8±5
G86	Gamed (dairy product)	70.4±3.5
G87	Gamed (dairy product)	93.5±4.6
G88	Gamed (dairy product)	67.9±3.3
G89	Gamed (dairy product)	70.2±3.5
G90	Gamed (dairy product)	64.8±3.2
G91	Gamed (dairy product)	67.8±3.3
G92	Gamed (dairy product)	87.8±4.3
G93	Gamed (dairy product)	53.1±2.6
G94	Gamed (dairy product)	70.2±3.5
G95	Gamed (dairy product)	69.2±3.4
SH96	Shanklish (dried Labanah traditional fermented by fungi)	85.7±4.2
SH97	Shanklish (dried Labanah traditional fermented by fungi)	89.4±4.4
SH98	Shanklish (dried Labanah traditional fermented by fungi)	92.5±4.6
SH99	Shanklish (dried Labanah traditional fermented by fungi)	90.8±4.5
SH100	Shanklish (dried Labanah traditional fermented by fungi)	89.9±4.4
SH101	Shanklish (dried Labanah traditional fermented by fungi)	75.1±3.7
SH102	Shanklish (dried Labanah traditional fermented by fungi)	79.1±3.9
SH103	Shanklish (dried Labanah traditional fermented by fungi)	83.6±4.1
SH104	Shanklish (dried Labanah traditional fermented by fungi)	85.8±4.2
SH105	Shanklish (dried Labanah traditional fermented by fungi)	86.6±4.3



Appendix C.1: Yeast strains proliferation (A), pH (B) and titratable acidity (C) of the non-fermented and fermented date pulp residues by *S. cerevisiae* and *P. kudriavzevii* during at 25 °C for 6 days.

Values are the means (n=3).

Error bars express standard deviation.

Appendix C.2: Relative peak area (RPA%) and peak areas (x105) of main volatile compounds in non-fermented (control) and fermented date pulp residues by *S. cerevisiae* and *P. kudriavzevii.*

		S. cerevisiae		***P. kudriavzevii***		**Control**	
Compounds	**RT**	**Peak Area**		**Peak Area**		**Peak Area**	
Acids							
4-Methyl-2-oxovaleric acid	19.67	0.15	±0.05	0.00	0.00	0.00	0.00
Acetic acid	19.76	1.68	±0.23	2.10	±0.31	1.42	±0.23
Isobutanoic acid	22.60	0.14	±0.02	0.41	±0.07	0.06	±0.02
Butanoic acid	24.09	0.10	±0.02	0.06	±0.01	0.03	±0.01
2-Methylbutanoic acid	25.03	0.10	±0.02	0.59	±0.10	0.12	±0.02
Hexanoic acid	28.92	1.29	±0.11	0.22	±0.01	0.06	±0.03
Heptanoic acid	31.13	0.13	±0.00	0.10	±0.02	0.12	±0.02
Octanoic acid	33.24	1.17	±0.17	0.26	±0.09	0.12	±0.01
Nonanoic acid	35.27	0.52	±0.24	0.72	±0.26	0.17	±0.05
Benzoic acid	40.21	0.11	±0.06	0.08	±0.01	0.03	±0.02
Subtotal		**5.40**		**4.55**		**2.14**	
RPA (%)		**1.26**		**1.60**		**1.92**	
Alcohols							
Isobutanol	9.63	1.69	±0.24	2.53	±0.29	0.00	0.00
Isoamyl alcohol	12.95	38.26	±9.23	30.21	±2.50	7.74	±1.28
1-Hexanol	17.16	0.24	±0.11	0.17	±0.10	0.27	±0.04
2-Ethyl-1-hexanol	21.02	1.29	±0.39	0.79	±0.10	0.94	±0.46
2-Phenylethanol	30.95	134.23	±15.35	46.97	±4.58	2.85	±1.59
2-Furanmethanol	25.18	0.10	±0.01	0.07	±0.01	0.13	±0.03
Subtotal		**175.82**		**80.74**		**11.94**	
RPA (%)		**40.99**		**28.33**		**10.72**	
Aldehydes							
Benzaldehyde	21.97	2.06	±0.79	5.95	±1.78	0.14	±0.05
Benzeneacetaldehyde	24.82	0.56	±0.16	0.27	±0.02	0.18	±0.07
4-Methylbenzaldehyde	25.09	0.14	±0.03	0.14	±0.02	0.00	±0.00
2,4-Dimethylbenzaldehyde	28.88	1.87	±0.08	1.60	±0.85	0.17	±0.01
Subtotal		**4.64**		**7.96**		**0.49**	
RPA (%)		**1.08**		**2.79**		**0.44**	
Esters							
Ethyl acetate	5.33	74.65	±14.03	92.28	±27.27	36.19	±14.57
Ethyl propanoate	6.33	1.76	±0.16	3.28	±0.26	1.50	±0.16
Isoamyl acetate	9.97	0.61	±0.26	1.17	±0.20	0.00	±0.00

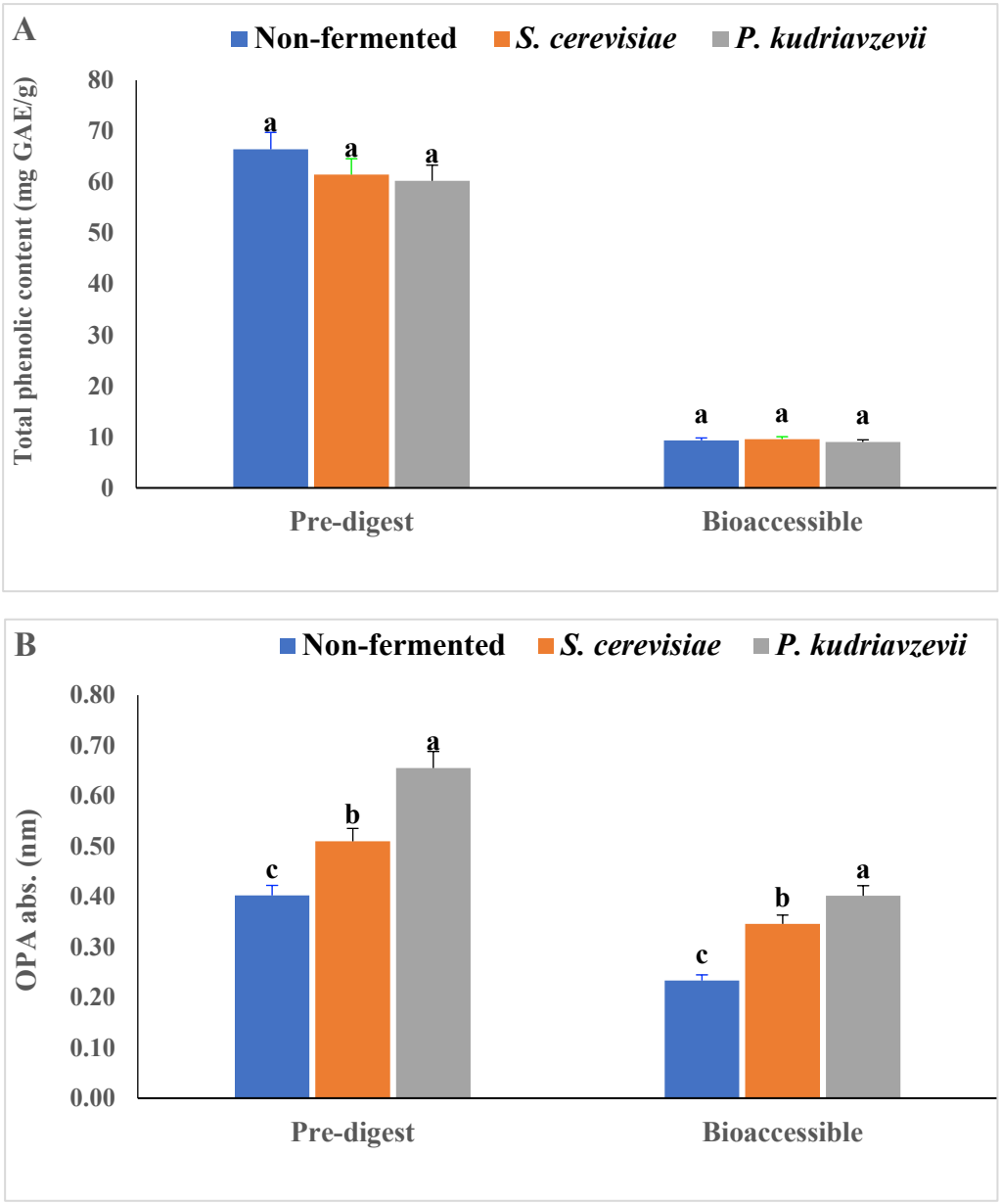
Value = mean ± standard deviation (SD), n=3

RPA (%) = (Base peak area/total) × 100

Appendix C.2: Relative peak area (RPA%) and peak areas (x105) of main volatile compounds in non-fermented (control) and fermented date pulp residues by *S. cerevisiae* and *P. kudriavzevii* (Continued).

Compounds	RT	*S. cerevisiae* Peak Area	*P. kudriavzevii* Peak Area	Control Peak Area
tert-Butyl formate	15.96	0.03 ±0.01	0.03 ±0.01	0.04 0.00
Ethyl octanoate	19.07	0.13 ±0.01	0.00 0.00	0.00 0.00
Ethyl decanoate	24.26	0.07 ±0.01	0.00 0.00	0.00 0.00
Phenethyl acetate	28.53	0.52 ±0.13	0.52 ±0.19	0.22 0.03
Methyl salicylate	27.93	0.14 ±0.05	0.08 ±0.01	0.08 0.00
Subtotal		**77.90**	**97.36**	**38.02**
RPA (%)		**18.16**	**34.16**	**34.14**
Ketones				
Acetone	4.73	1.30 ±0.27	2.19 ±1.02	3.86 ±0.75
2-Methyl-3-thiolanone	21.86	0.03 0.00	0.00 0.00	0.00 0.00
Butyrolactone	24.71	0.03 ±0.01	0.03 0.00	0.00 0.00
Subtotal		**1.37**	**2.22**	**3.86**
RPA (%)		**0.32**	**0.78**	**3.47**
Phenols				
2,4-Di-tert-butylphenol	37.88	78.89 ±12.22	61.03 ±7.14	46.24 ±9.30
2-Acetyl-4-methylphenol	36.24	0.10 ±0.02	0.00 0.00	0.00 0.00
t-Butylhydroquinone	62.17	0.12 ±0.02	0.10 ±0.02	0.09 ±0.02
Guaiacol	29.59	0.02 0.00	0.01 0.00	0.01 ±0.01
Subtotal		**79.13**	**61.14**	**46.35**
RPA (%)		**18.45**	**21.45**	**41.62**
Others				
Dimethyl ether	5.960	84.23 ±41.32	30.60 ±12.57	8.05 ±3.15
2-Acetylfuran	21.469	0.11 ±0.03	0.11 0.00	0.13 ±0.01
1H-Indene, octahydro-2,2,4,4,7,7-hexamethyl-, *trans*-	26.735	0.03 ±0.003	0.16 ±0.052	0.17 ±0.044
Naphthalene	27.214	0.18 ±0.01	0.10 ±0.02	0.11 ±0.04
Isobutyric anhydride	29.685	0.05 ±0.01	0.02 ±0.01	0.02 ±0.01
1-Phenylpropane-1,2-diol	30.156	0.03 0.00	0.02 0.00	0.02 0.00
3-Acetyl-1H-pyrroline	31.938	0.05 ±0.01	0.04 ±0.01	0.05 0.00
Subtotal		**84.69**	**31.06**	**8.56**
RPA (%)		**19.74**	**10.90**	**7.69**
Total		**428.94**	**285.03**	**111.36**

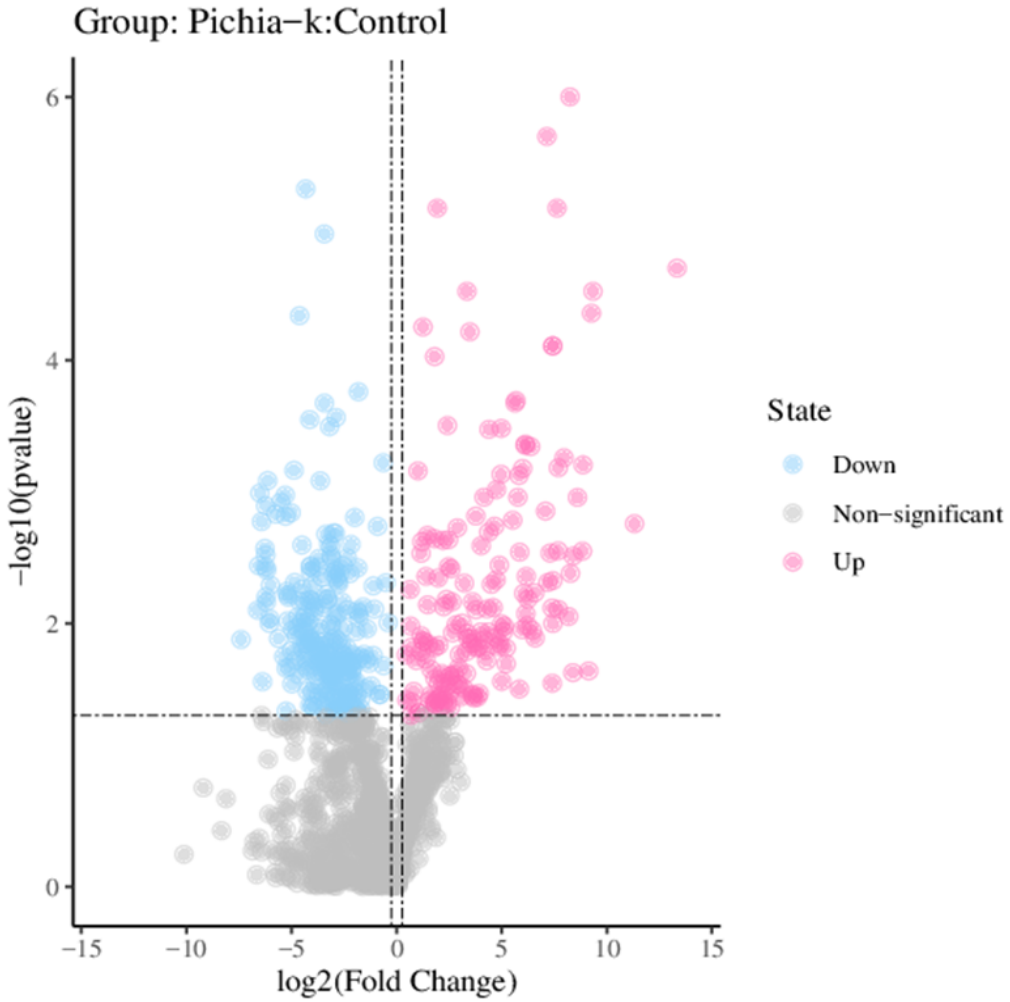
Value = mean ± standard deviation (SD), n=3
RPA (%) = (Base peak area/total) × 100



Appendix C.3: Total phenolic compounds (A) and OPA absorbances (B) of undigested samples and bioaccessible portion of non-fermented (control) and fermented date pulp residues by *S. cerevisiae* and *P. kudriavzevii*.

Values are means (n=3). Values are the means (n=3).

Error bars express standard deviation.



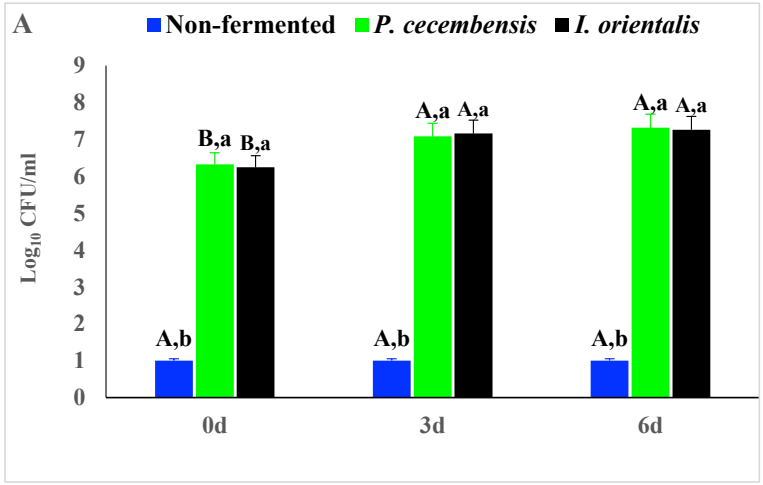
Appendix C.4: A volcano map displays the differing metabolites between non-fermented and fermented date pulp residues by *P. kudriavzevii.*

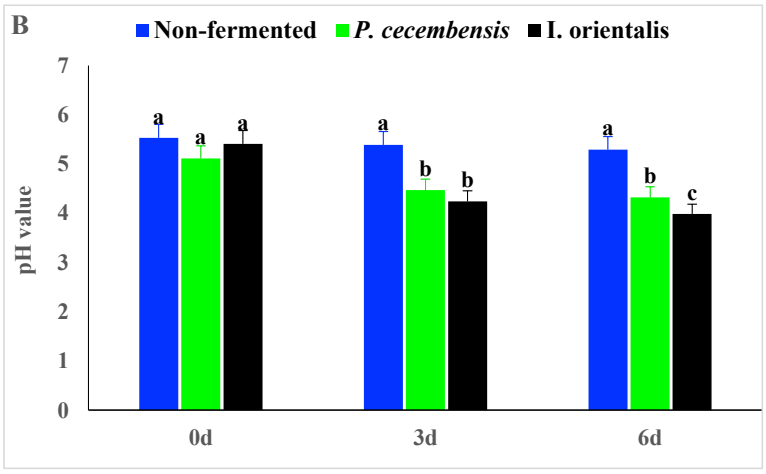
Appendix C.5: Top ten enriched metabolic pathways with identified metabolites.

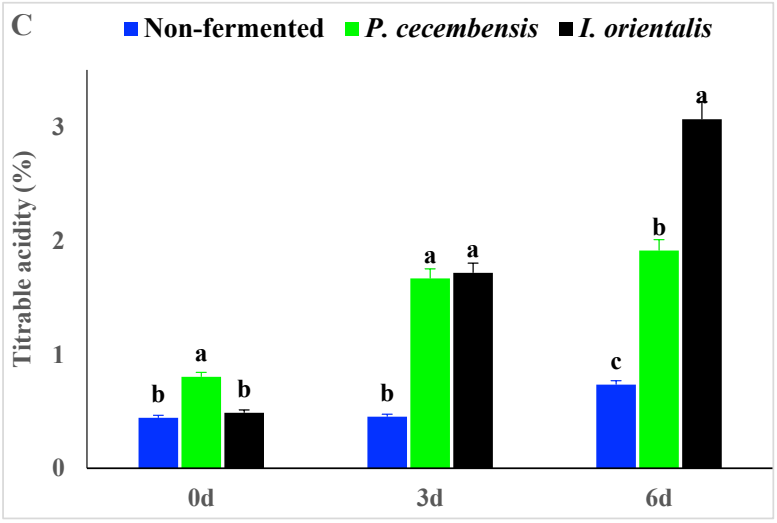
Pathway	**KEGG Names**
2-Oxocarboxylic acid metabolism	L-Glutamic acid Oxoglutaric acid L-Methionine L-Isoleucine L-Tryptophan 2-Isopropylmalic acid (-)-threo-isodihomocitric acid L-(+)-Valine (1*E*)-*N*-Hydroxy-4-(methylsulfanyl)-1-butanimine 4-Methylthiobutylthiohydroximate (*E*)-5-(methylsulfanyl)pentanal oxime
Biosynthesis of amino acids	L-Glutamic acid Oxoglutaric acid L-arginine L-Methionine L-Isoleucine L-Tryptophan 2-Isopropylmalic acid Shikimate D-(-)-3-Phosphoglyceric acid L-(+)-Valine
ABC transporters	L-Glutamic acid L-arginine L-Isoleucine a-Lactose Xylitol 1,4-D-xylobiose Glycerin L-(+)-Valine Phthalic acid
Aminoacyl-tRNA biosynthesis	L-Glutamic acid L-arginine L-Methionine L-Isoleucine L-Tryptophan L-(+)-Valine
Glucosinolate biosynthesis	L-Methionine L-Isoleucine L-Tryptophan

Appendix C.5: Top ten enriched metabolic pathways with identified metabolites (Continued).

Pathway	**KEGG Names**
Glucosinolate biosynthesis	L-(+)-Valine (1*E*)-*N*-Hydroxy-4-(methylsulfanyl)-1-butanimine 4-Methylthiobutylthiohydroximate (*E*)-5-(methylsulfanyl)pentanal oxime
Tryptophan metabolism	L-Tryptophan 4-Hydroxy-2-quinolinecarboxylic acid Indole-3-acetic acid Indole-3-ethanol Skatole Indole 5-(3'-carboxy-3'-oxopropyl) -4,6-dihydroxypicolinate
Butanoate metabolism	(R)-Malate L-Glutamic acid Oxoglutaric acid Biacetyl (+/-)-2-Hydroxyglutaric acid
Plant hormone signal transduction	N6-(delta-2-isopentenyl)-adenine Indole-3-acetic acid Salicylic acid
Pentose and glucuronate interconversions	Oxoglutaric acid L-arabitol Ribitol Xylitol Glycerin
C5-Branched dibasic acid metabolism	Itaconate L-Glutamic acid Oxoglutaric acid (+/-)-2-Hydroxyglutaric acid







Appendix D.1: Yeast strains population (A), pH (B) and titratable acidity (C) of the unfermented and fermented date syrup waste by *P. cecembensis* and *I. orientalis* during 6 days at 25°C.

Value = mean of triplicates.

Standard deviation (SD) is displayed in error bars.

Appendix D.2: Peak areas (x105) and relative peak area (RPA%) of selected volatiles in non-fermented (control) and fermented date syrup waste by *P. cecembensis* and *P. kudriavzevii*.

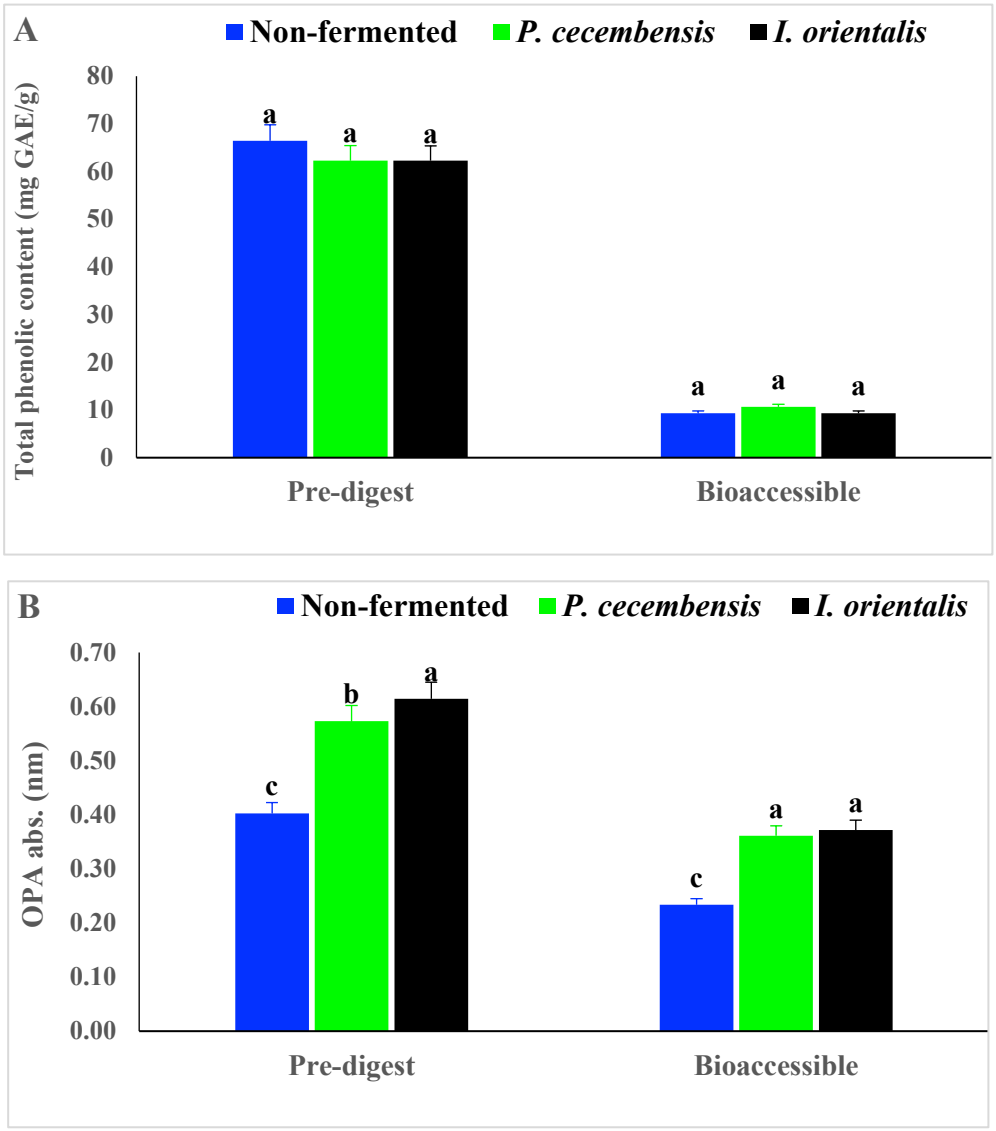
Compounds	RT	*P. cecembensis*		*P. kudriavzevii*		Non-fermented	
		Peak Area		Peak Area		Peak Area	
Acids							
4-Methyl-2-oxovaleric acid	19.67	0.23	±0.04	0.00	±0.00	0.00	±0.00
Acetic acid	19.76	1.09	±0.23	1.64	±0.84	1.42	±0.23
Isobutanoic acid	22.6	0.15	±0.01	0.52	±0.21	0.06	±0.02
Butanoic acid	24.09	0.05	±0.02	0.04	±0.02	0.03	±0.01
2-Methylbutanoic acid	25.03	0.15	±0.04	0.73	±0.23	0.12	±0.02
Hexanoic acid	28.92	0.77	±0.15	0.23	±0.09	0.06	±0.03
Heptanoic acid	31.13	0.11	±0.01	0.14	±0.01	0.12	±0.02
Octanoic acid	33.24	0.54	±0.19	0.24	±0.11	0.12	±0.01
Nonanoic acid	35.27	0.58	±0.15	0.34	±0.14	0.17	±0.05
Benzoic acid	40.21	0.20	±0.12	0.07	±0.03	0.03	±0.02
Subtotal		**3.87**		**3.95**		**2.14**	
RPA (%)		**1.04**		**1.02**		**1.92**	
Alcohols							
Isobutanol	9.63	1.41	±0.31	2.56	±0.45	0	±0.00
Isoamyl alcohol	12.95	30.33	±4.44	29.52	±2.73	7.74	±1.28
1-Hexanol	17.16	0.12	±0.04	0.12	±0.02	0.27	±0.04
2-Ethyl-1-hexanol	21.02	1.23	±0.21	0.94	±0.21	0.94	±0.46
2-Phenylethanol	30.95	109.77	±9.46	37.91	±9.00	2.85	±1.59
2-Furanmethanol	25.18	0.07	±0.02	0.09	±0.02	0.13	±0.03
Subtotal		**142.94**		**71.15**		**11.94**	
RPA (%)		**38.52**		**18.41**		**10.72**	
Aldehydes							
Benzaldehyde	21.97	1.69	±0.65	1.62	±0.43	0.14	±0.05
Benzeneacetaldehyde	24.82	0.48	±0.07	0.22	±0.01	0.18	±0.07
4-Methylbenzaldehyde	25.09	0.18	±0.01	0.13	±0.05	0.00	±0.00
2,4-Dimethylbenzaldehyde	28.88	1.19	±0.09	1.95	±0.35	0.17	±0.01
Subtotal		**3.54**		**3.92**		**0.49**	
RPA (%)		**0.95**		**1.01**		**0.44**	
Ketones							
Acetone	04.73	02.21	±0.44	01.6	±0.26	3.86	±0.75
2-Methyl-3-thiolanone	21.86	00.05	±0.01	0.00	±0.00	0.00	±0.00
Butyrolactone	24.71	00.04	±0.01	0.03	±0.01	0.00	±0.00
Subtotal		**2.29**		**1.63**		**3.86**	
RPA (%)		**0.62**		**0.42**		**3.47**	
Esters							
Ethyl acetate	5.33	98.79	±15.09	200.85	±81.6	36.19	±14.6
Ethyl propanoate	6.33	0.00	±0.00	2.56	±0.35	1.5	±0.16

Value = mean of triplicates ± standard deviation (SD)
RPA (%) = (peak area/total peak area) × 100

Appendix D.2: Peak areas (x105) and relative peak area (RPA%) of selected volatiles in non-fermented (control) and fermented date syrup waste by P. cecembensis and P. kudriavzevii (Continued).

Compounds	RT	*P. cecembensis* Peak Area		*P. kudriavzevii* Peak Area		Non-fermented Peak Area	
Isoamyl acetate	09.97	0.92	±0.47	2.13	±0.54	0.00	±0.00
tert-Butyl formate	15.96	0.02	±0.01	0.02	±0.01	0.00	±0.04
Ethyl octanoate	19.07	0.22	±0.06	0.00	±0.00	0.00	±0.00
Ethyl decanoate	24.26	0.13	±0.05	0.04	±0.02	0.00	±0.00
Phenethyl acetate	28.53	1.19	±0.42	0.69	±0.09	0.22	±0.03
Methyl salicylate	27.93	0.16	±0.03	0.1	±0.02	0.08	±0.00
Subtotal		**101.42**		**206.39**		**38.02**	
RPA (%)		**27.33**		**53.41**		**34.14**	
Phenols							
2,4-Di-tert-butylphenol	37.88	86.18	±2.57	75.2	±7.30	46.24	±9.30
2-Acetyl-4-methylphenol	36.24	0.12	±0.04	0.00	±0.00	0.00	±0.00
t-Butylhydroquinone	62.17	0.19	±0.02	0.12	±0.02	0.09	±0.02
Guaiacol	29.59	0.02	0.00	0.02	±0.00	0.01	±0.01
Subtotal		**86.51**		**75.34**		**46.35**	
RPA (%)		**23.32**		**19.5**		**41.62**	
Others							
Dimethyl ether	5.96	30.04	±15.0	23.6	±9.36	8.05	±3.15
2-Acetylfuran	21.47	0.10	±0.01	0.08	±0.01	0.13	±0.01
1H-Indene, octahydro-2,2,4,4,7,7-hexamethyl-trans-	26.74	0.08	±0.04	0.17	±0.03	0.18	±0.04
Naphthalene	27.21	0.13	±0.03	0.13	±0.01	0.11	±0.04
Isobutyric anhydride	29.68	0.05	±0.01	0.03	±0.00	0.02	±0.01
1-Phenylpropane-1,2-diol	30.16	0.03	±0.00	0.03	±0.00	0.02	0.00
3-Acetyl-1H-pyrroline	31.94	0.05	±0.01	0.04	±0.01	0.05	0.00
Subtotal		**30.49**		**24.07**		**8.56**	
RPA (%)		**08.22**		**06.23**		**06.23**	
Total peak area		**371.05**		**386.44**		**111.36**	

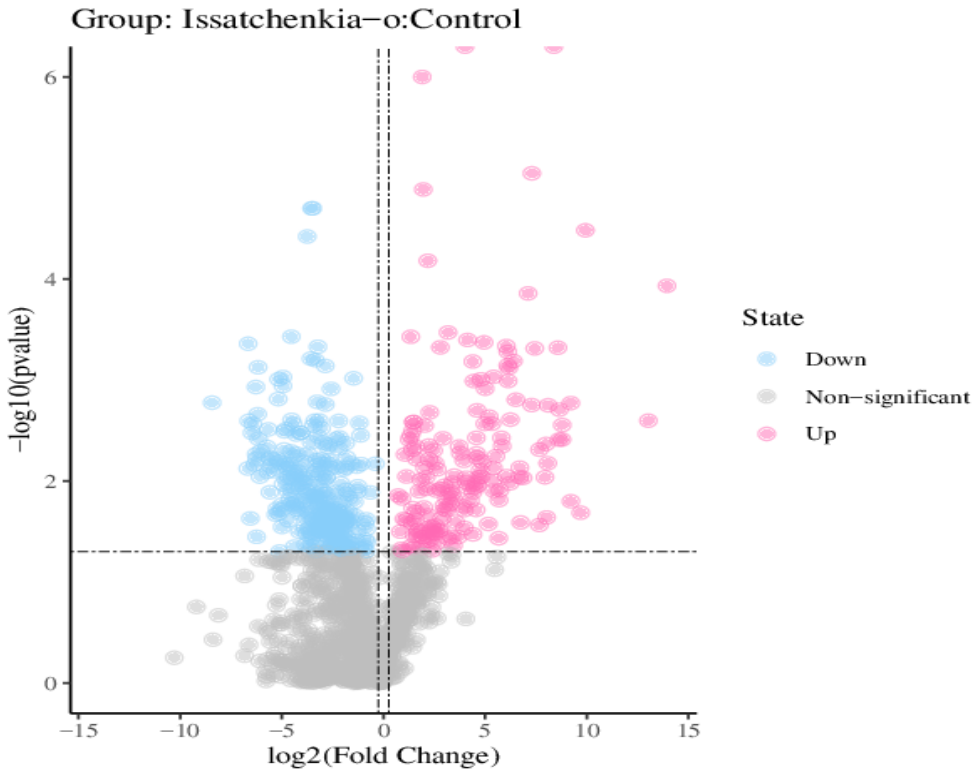
Value = mean of triplicates ± standard deviation (SD)
RPA (%) = (peak area/total peak area) × 100



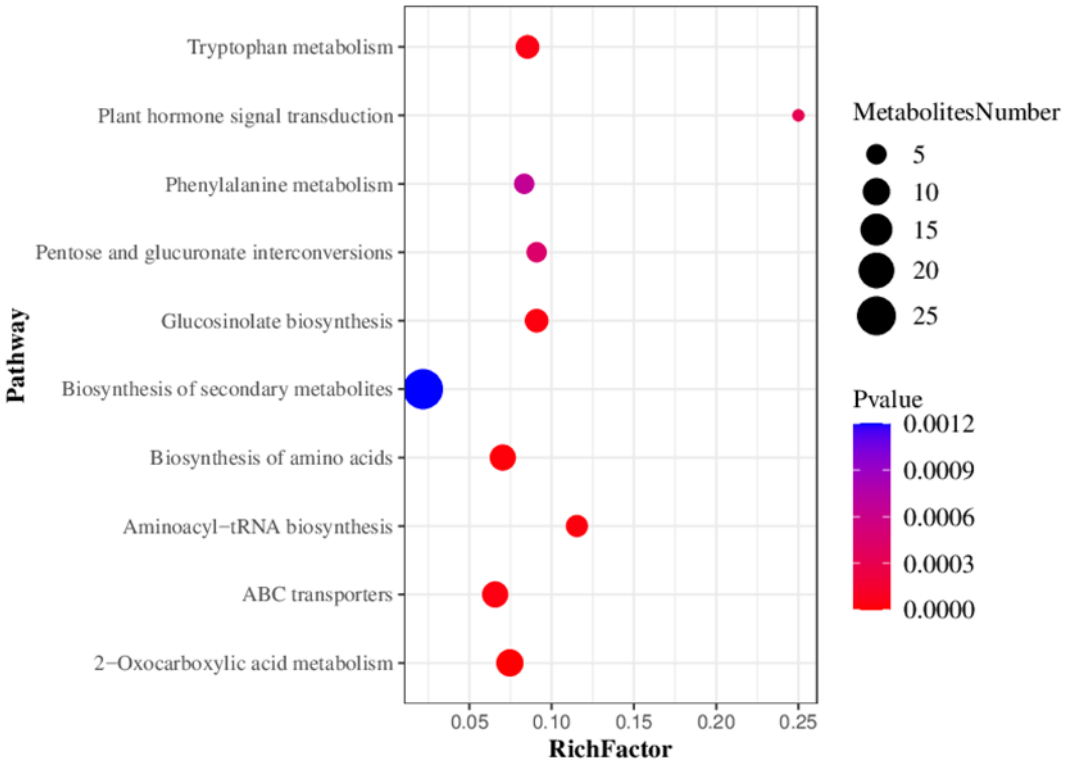
Appendix D.3: Total phenolic contents (A) and OPA absorbances (B) of non-digestible samples and bioaccessible portion of un-fermented (control) and fermented date syrup waste by *P. cecembensis* and *I. orientalis*.

Value = mean of triplicates.

Standard deviation (SD) is displayed in error bars.



Appendix D.4: Volcanic map of differential metabolites between fermented date syrup waste by *I. orientalis* and control.



Appendix D.5: Metabolic pathway enrichment analysis of differential metabolites between fermented date syrup waste by *P. kudriavzevii* and control.

The RichFactor on the X-axis represents the ratio of differential metabolites annotated in a pathway. The dot size indicates the number of differential metabolites annotated in that pathway.

Appendix D.6: Top 10 enriched metabolic pathways and their metabolites.

Pathway	KEGG Names	Status
2-Oxocarboxylic acid metabolism	L-Glutamic acid	upregulated
	Oxoglutaric acid	downregulated
	L-Methionine	upregulated
	L-Isoleucine	upregulated
	L-Tryptophan	upregulated
	2-Isopropylmalic acid	upregulated
	L-(+)-Valine	upregulated
	(1E)-N-Hydroxy-4-(methylsulfanyl)-1-butanimine	downregulated
	4-Methylthiobutylthiohydroximate	downregulated
	(*E*)-5-(methylsulfanyl)	downregulated
Biosynthesis of amino acids	L-Glutamic acid	upregulated
	Oxoglutaric acid	downregulated
	L-Arginine	upregulated
	L-Methionine	upregulated
	L-Isoleucine	upregulated
	L-Tryptophan	upregulated
	2-Isopropylmalic acid	upregulated
	D-(-)-3-Phosphoglyceric acid	downregulated
	L-(+)-Valine	upregulated
Aminoacyl-tRNA biosynthesis	L-Glutamic acid	upregulated
	L-Arginine	upregulated
	L-Methionine	upregulated
	L-Isoleucine	upregulated
	L-Tryptophan	upregulated
	L-(+)-Valine	upregulated
Glucosinolate biosynthesis	L-Methionine	upregulated
	L-Isoleucine	upregulated
	L-Tryptophan	upregulated
	L-(+)-Valine	upregulated
	(1*E*)-*N*-Hydroxy-4-(methylsulfanyl)-1-butanimine	downregulated
	4-methylthiobutylthiohydroximate	downregulated
	(*E*)-5-(methylsulfanyl)pentanal oxime	downregulated
ABC transporters	L-Glutamic acid	upregulated
	L-Arginine	upregulated
	L-Isoleucine	upregulated
	a-Lactose	upregulated
	Xylitol	upregulated
	1,4-D-xylobiose	downregulated
	Glycerin	upregulated
	L-(+)-Valine	upregulated
	Phthalic acid	upregulated
Tryptophan metabolism	L-Tryptophan	upregulated
	4-Hydroxy-2-quinolinecarboxylic acid	downregulated
	Indole-3-acetic acid	
	Indole-3-ethanol	upregulated

Appendix D.6: Top 10 enriched metabolic pathways and their metabolites (Continued).

Pathway	KEGG Names	Status
	Skatole	upregulated
	Indole	upregulated
	5-(3'-Carboxy-3'-oxopropyl) -4,6-dihydroxypicolinate	downregulated
Plant hormone signal transduction	N6-(delta-2-isopentenyl)-adenine	downregulated
	Indole-3-acetic acid	downregulated
	Salicylic acid	upregulated
Pentose and glucuronate interconversions	Oxoglutaric acid	downregulated
	L-arabitol	upregulated
	Ribitol	upregulated
	Xylitol	upregulated
	Glycerin	upregulated
Phenylalanine metabolism	Succinate	downregulated
	2-Hydroxycinnamic acid	upregulated
	Salicylic acid	upregulated
	trans-Cinnamate	upregulated
	Benzoic acid	upregulated
Biosynthesis of secondary metabolites	L-Glutamic acid	upregulated
	Oxoglutaric acid	downregulated
	Succinate	downregulated
	L-Arginine	upregulated
	L-Methionine	upregulated
	L-Isoleucine	upregulated
	L-Tryptophan	upregulated
	N6-(delta-2-isopentenyl)-adenine	downregulated
	2-Hydroxycinnamic acid	upregulated
	2-Isopropylmalic acid	upregulated
	4-Coumaric acid	upregulated
	Salicylic acid	upregulated
	Mevalonic acid	upregulated
	trans-Cinnamate	upregulated
	Pantothenic acid	upregulated
	12-Oxo-phytodienoic acid	upregulated
	Senecionine	downregulated
	Coenzyme	upregulated
	D-(-)-3-Phosphoglyceric acid	downregulated
	L-(+)-Valine	upregulated
	Benzoic acid	upregulated
	(1*E*)-N-Hydroxy-4-(methylsulfanyl)-1-butanimine	downregulated
	Indole	upregulated

Appendix D.6: Top 10 enriched metabolic pathways and their metabolites (Continued).

Pathway	KEGG Names	Status
Biosynthesis of secondary metabolites	4-Methylthiobutylthiohydroximate	downregulated
	Traumatin	downregulated
	(2*E*,6*E*)-Farnesol	downregulated
	(-)-Lupinine	Downregulated

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The probiotic properties of yeast strains isolated from food sources were characterized, followed by the investigation of the bioactive properties of date palm pomace (DPP) through fermentation with novel probiotic yeast candidates. This dissertation explores the potential of utilizing agricultural by-products, such as DPP, to advance sustainable food production, presenting a promising opportunity.

Nadia Al Kalbani received her PhD in Food Science and Technology from the Department of Food Science, Agriculture & Veterinary Medicine at UAE University, UAE. She received her Master of Science in Food Science from the College of Agriculture & Veterinary Medicine, UAE University, UAE.

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