U.PORTO
FEUP FACULDADE DE ENGENHARIA
UNIVERSIDADE DO PORTO

lepabe
Laboratory for Process Engineering,
Environment, Biotechnology and Energy

Master Thesis

Bioengineering Master – Specialization in Biological Engineering

2021/2022

Table olive fermentation using a potentially probiotic lactic acid bacterium strain: effects upon microbiological, physicochemical, nutritional, and sensory profiles of final product

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Under the auspices of PROMETHEUS (FCT) project,

coordinated by Professor Doutor F. Xavier Malcata

Porto, 23rd September 2022

"On ne voit bien qu'avec le cœur,
l'essentiel est invisible pour les yeux."

Antoine de Saint-Exupéry

ACKNOWLEDGMENT

I would first like to thank my thesis supervisor, Dr. Ângela Macedo, and co-supervisor, Dr. Patrícia Reis, besides Dr. Tânia Tavares, for the so many hours spent, either in the laboratory or in video meetings, and for the shared knowledge, experience, guidance, and support. I must also thank to Prof. Dr. F. Xavier Malcata, for showing me a way to my future, by revealing to me the area I wanted to professionally pursue via his courses that I attended for the past 5 years, and for steering me into his research group – where I had great pleasure to work, further to making this work possible. I would also like to acknowledge all the professors, researchers and technicians that were somehow involved in my academic journey. Also, I would like to thank Prof. Dr. Romualdo Salcedo for providing us storage space and the possibility to perform the experiments.

I would also like to thank Casa Agrícola Quinta da Cruzinha Lda (Mosteiró, Torre D. Chama, Mirandela) who provided the samples of *Cobrançosa* table olives for this work, in special to Eng. Bruno Costa for his time and availability for the logistic arrangements underlying sample supply; as well as to the panellists, who filled in the sensory survey for this research project. Without their participation and input, the whole work would be handicapped.

The Bromatology and Hydrology Laboratory of the Chemical Sciences Department of Faculty of Farmacy of University of Porto is gratefully acknowledged for availability and collaboration in several nutritional analysis required by this work, namely Dr. Eulália Mendes and Dr. Susana Casal who guided me in through the laboratory techniques.

Finally, I must express my very deep gratitude to my parents, siblings, uncles, cousins, and my dear boyfriend – not forgetting all my colleagues and friends, for providing me unfailing support and continuous encouragement throughout all these years of study, and through the process of research development and writing of this thesis. This accomplishment would not have been possible without them. I would also like to specially acknowledge my colleague and friend Afonso Silva, who accompanied me in most steps of the laboratory work.

This work was financially supported by: LA/P/0045/2020 (ALiCE), UIDB/00511/2020 and UIDP/00511/2020 (LEPABE), funded by national funds through FCT/MCTES (PIDDAC); and Project - PROMETHEUS-POCI-01-0145-FEDER-029284, funded by FEDER funds through COMPETE2020 – Programa Operacional Competitividade e Internacionalização (POCI) and by national funds (PIDDAC) through FCT/MCTES.

For the accomplishment of this work, I was financially supported by a Research Grant, under Partnership Agreement with Faculty of Engineering of University of Porto, inserted in project PROMETHEUS-POCI-01-0145-FEDER-029284.



RESUMO

As azeitonas de mesa são um dos constituintes mais importantes da dieta Mediterrânica, tendo o seu consumo vindo a aumentar ano após ano. Contudo, o processamento natural das azeitonas, por fermentação espontânea, é ainda praticado de forma empírica e tradicional, contribuindo para a aleatoriedade dos seus parâmetros físico-químicos e microbiológicos. No presente trabalho, foram avaliadas diferentes estratégias de inoculação de *Lactiplantibacillus pentosus* i106 - uma estirpe de bactéria lática previamente selecionada pelo seu potencial probiótico, e evitando interferência significativa no processo de fermentação natural da azeitona de mesa *Cobrançosa* (Trás-os-Montes, Portugal), em (A) azeitonas da colheita de 2020/21, com concentrações de NaCl de 0, 3, 5, e 7%, sem fase do adoçamento; (B) em azeitonas da colheita de 2020/21, com 5 e 7% de NaCl, durante o processo de salga e sem interferência na fase do adoçamento; e (C) em azeitonas da colheita de 2019/20 prontas a consumir com 5% de NaCl, e sem interferência nas fases de adoçamento e salga. Durante a fermentação, as alterações microbiológicas e físico-químicas foram monitorizadas, e as propriedades nutricionais e sensoriais finais foram avaliadas. Os resultados indicaram que: na colheita de 2020/21, as bactérias láticas predominam aquando da inoculação, enquanto as leveduras predominam no controlo e mantêm-se constantes ao longo do tempo. Na colheita de 2019/20, os microrganismos apresentaram níveis semelhantes; contudo, verificou-se que o crescimento de bactérias láticas é prejudicado por percentagens elevadas de sal (>5%). Além disso, mostrou-se que a inibição do crescimento de *Enterobacteriaceae* pode ser facilitado pela diminuição de pH, aumento de acidez e atividade das bactérias láticas, mesmo sob baixas concentrações de sal. Apesar de a degradação de compostos fenólicos na salmoura, nomeadamente oleuropeína, não ter ocorrido gradualmente como esperado, esta foi alcançada mais rapidamente após inoculação de *Lpb. pentosus* i106 - produzindo níveis mais elevados de hidroxitirosol e verbascosídeo, ou ainda tirosol. Nutricionalmente, tal como esperado, a colheita de 2020/21 (azeitona mista) apresentou maior teor de gordura e menor humidade face à de 2019/20 (azeitona verde), pois a colheita nem sempre é feita no mesmo estado de maturação; já entre corridas de cada plano, não foram encontradas diferenças significativas. Notavelmente, a inoculação em momentos com níveis de sal diferentes resultou em características organoléticas distintas - concluindo-se assim que a inoculação da azeitona com níveis reduzidos de NaCl é viável (e desejável). Considerando a qualidade geral de consumo, inferiu-se que o melhor protocolo é aquele usado pelo produtor (plano C), e que a estirpe *Lpb. pentosus* i106 pode realmente ser adicionada e conferiu alguns benefícios, mas como aditivo no fim da fermentação e não como cultura de arranque.

Palavras - chave: azeitonas de mesa; bactérias do ácido lático; probióticos; fermentação; cultura de arranque; alterações microbiológicas; alterações físico-químicas; análise nutricional; análise sensorial

ABSTRACT

Table olives are one of the most important constituents of the Mediterranean diet, and have undergone a significant increasing consumption year after year. However, table olive natural-style processing, by spontaneous fermentation, is still performed empirically following traditional practices, which contributes to the randomness of physicochemical and microbiological parameters. In the present work, alternative strategies were evaluated for inoculation of *Lactiplantibacillus pentosus* i106 - a lactic acid bacteria strain previously selected for its probiotic potential, while avoiding significant interference in the natural process of *Cobrançosa* table olives (Trás-os-Montes, Portugal): (A) table olives from the 2020/21 harvest, with NaCl concentrations of 0, 3, 5, and 7%, without sweetening; (B) table olives from the 2020/21 harvest, with 5 and 7% of NaCl, during the stage of salting without interference on sweetening; and (C) ready-to-eat table olives from the 2019/20 harvest with 5% of NaCl, without interference on sweetening and salting processes. During fermentation, microbiological and physicochemical alterations were monitored, and final nutritional and sensory properties were evaluated. This study results indicated that in the 2020/21 harvest, upon inoculation, LAB are predominant, while yeasts predominate in control and remain constant through time; in 2019/20 harvest, microorganisms were found to similar levels; however, the LAB strain growth is hindered by high salt percentages (above 5%). In addition, the inhibition of enterobacterium growth is facilitated by low pH, high acidity, and LAB strain activity. Even though phenolic compound degradation, namely oleuropein, did not occur gradually as expected, it occurred faster in inoculated trials, thus producing higher levels of hydroxytyrosol and verbascoside, or even tyrosol in case of plan A. Nutritionally speaking, and as expected, the 2020/21 harvest presented higher fat content and lower water content when compared to the 2019/20 harvest - justified by the fact the table olive harvest is not always performed at the same stage of maturation; on the other hand, no significant differences were found among trials from each protocol. Notably, different salt levels and inoculation moments resulted in distinct sensory properties, so table olive production with reduced NaCl content is feasible (and desired). Considering the overall eating quality, it can be inferred that the best protocol is that used by the producer (plan C) and that, in fact, strain *Lpb. pentosus* i106 can be added and provide benefits, but as an additive at the end of fermentation rather than as a starter culture.

Keywords: table olives; lactic acid bacteria; probiotics; fermentation; starter culture; microbiological changes; physicochemical changes; nutritional analysis; sensory analysis.

DECLARATION

I pledge my honour that this work is original and that any other contributions were dully referenced with identification of the source.

Declaro, sob compromisso de honra, que este trabalho é original e que todas as contribuições não originais foram devidamente referenciadas com identificação da fonte.

23rd September



(Joana Coimbra Silva Marques Gomes)

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LIST OF ABBREVIATIONS

ANOVA Analysis of Variance

AOAC Association of Official Analytic Chemists

CFU Colony-forming Unit

cv. Cultivar

CVr Robust Coefficient of Variation

FAO Food and Agriculture Administration

HPLC High-performance Liquid Chromatography

IOC International Olive Council

LAB Lactic Acid Bacteria

MRS Man Rogosa and Sharpe

NSC Natural Starter Cultures

OD Optical Density

RBC Rose Bengal Chloramphenicol

SSC Selected Starter Cultures

TTA Total Titratable Acidity

VRBGA Violet Red Bile Glucose Agar

WHO World Health Organization

I. INTRODUCTION

1. Contextualization

In order to remain competitive in the global market, agri-food industries have been struggling for innovation in their processes and products - while trying to address the issues of environmental sustainability and food safety (Bigliardi and Filippelli, 2022). Food industry is growing towards not only a higher demand for quality food, but also food nutrients that can bring positive effects upon human health. Such "functional foods" are whole, fortified, enriched, or enhanced foods that provide health benefits beyond provision of essential nutrients (like vitamins and minerals) when consumed at sufficient levels, and as part of a balanced diet. In this context, probiotics can be defined as viable microorganisms (such as bacteria or yeasts) that, if ingested in appropriate quantity, can exert several beneficial effects upon human health; besides that, scientific evidence and solid data should support those health claims (Albuquerque *et al.*, 2019; Hasler, 2002; Koirala and Anal, 2021).

Traditional fermented foods are an important part of cultural heritage and diet, widely consumed since ancient civilizations - and used to preserve and increase the safety of existing food resources (Kavitake *et al.*, 2018). In the past, autochthonous bacteria (and sometimes yeasts) were traditionally used in food fermentation; nowadays, development of applied microbiology has led to selection of specific bacteria to be applied in specific food matrices, thus enhancing food technological and organoleptic properties (Sedó Molina *et al.*, 2022). There is a major window of opportunity lying in the field of novel probiotic strains – isolated from less-conventional natural sources, but able to confer advanced health promoting properties to the host upon regular and balanced administration via traditional foods; this includes adventitious strains isolated from plant matrices, in alternative to classical animal-based probiotic strains in dairy foods (Bulut Albayrak and Duran, 2022; De Bellis *et al.*, 2021). Comparing animal with plant-based fermented products market, the second has been increasing from year to year due to their higher sustainability, health-related benefits, lifestyle (e.g., vegans or vegetarians) or dietary reasons (e.g., lactose intolerance, concerns over cholesterol accumulation); however, it is still necessary to overcome a few sensory, technological and nutritional challenges (Perpetuini *et al.*, 2020; Sedó Molina *et al.*, 2022).

In this regard, the probiotic potential of native table olive strains from different countries, such as Greece, Italy, Spain, Cyprus, Morocco or Portugal, has been studied (Abouloifa *et al.*, 2020; Portilha-Cunha *et al.*, 2020). The World Catalogue of Olive Cultivars (IOC, 2013) reports about 2500 olive varieties - but only 10% can be considered commercial, for being used as oil, table olives or both. Fermented table olives, with major importance in the Mediterranean diet, are accordingly rank among the most popular and healthy fruits; this justifies their worldwide socioeconomic impact (Anagnostopoulos and Tsaltas, 2022). According to Food and Agriculture Organization (FAO, 2022), 96% of the world's production of olives occurs in Mediterranean countries. As reported by the International Olive Council (IOC, 2022), the world production from the 2019/20 campaign has increased 5.5% compared to the previous one; however, the 2020/21 campaign decreased 10.1% relative to previous year, probably due to the emerging drought around and the world, namely in Portugal. Among the IOC member countries, Spain stands out for its weight in world production (20.5%), 19.3% higher than the previous year; whereas Egyptian production has decreased by 23.1%, compared to the 2019/20 campaign, thus contributing 18.8% of the world total. Estimates for the 2021/22 campaign anticipate an increase by 7% compared to 2020/21 – thus reaching 2 846 500 ton, with consumption increasing by 1.2% (IOC, 2022).

In Portugal the production of table olives is estimated to have an increase of 34.6%, attaining a production of 21 100 ton/year (IOC, 2022). Portuguese cultivars, such as *Galega* (Peres *et al.*, 2014) and *Negrinha do Freixo* (Oliveira *et al.*, 2017; Pereira *et al.*, 2015), have already been studied regarding their functional properties and benefits; nevertheless, *Cobrançosa* table olive, obtained from the Northern Portugal, is one of the Portuguese table olive varieties whose endogenous microbiota has attracted recent interest for potentially probiotic features (Albuquerque *et al.*, 2019).

2. Table olive production

The major components of olives are water (60–75%), lipids (10–25%), reducing sugars (2–5%), and phenolic compounds (1–3%) (Conte *et al.*, 2020). The lipidic content, mainly unsaturated fat, is associated with cardiovascular disease prevention, since it favours the "good cholesterol" – HDL; but polyunsaturated fat also has relevance, and should be present in the diet, given the fact that it cannot be produced by the human organism (Prata, 2015). Unripe olives are not edible due to high content of phenolic

compounds, specially oleuropein, responsible for fruit bitterness. Hence, table olives must undergo a series of physicochemical and microbiological transformations via the traditional table olive processing based on fermentation in brine (Albuquerque *et al.*, 2019). On the other hand, the presence of these compounds confers antioxidant properties, due to the di-hydroxy radicals linked in an aromatic structure – and thus prevent cardiovascular diseases, some cancers, and inflammations (Prata, 2015).

According to the degree of ripeness, several types of table olives can be named, with preparation techniques varying from country to country (Albuquerque *et al.*, 2019; Benitez-Cabello *et al.*, 2020). Natural table olives (or Greek style for black olives) are placed directly in brine, typically solutions of 6-10% (w/v) NaCl (Campus *et al.*, 2018; Findik *et al.*, 2016); treated table olives (when applied to green olives, Spanish-style if fermentation occurs, or Picholine-style if 5–10% NaCl without fermentation) undergo alkaline treatment with sodium hydroxide, with controlled salt percentage and low pH to eliminate unwanted microorganisms and avoid deterioration (Rejano, 2010); darkened by oxidation table olives (Californian-style) are treated directly or preserved in brine, oxidized, washed, and packed in cans hermetically sealed and sterilized - so they do not undergo fermentation at all, while guaranteeing product safety and avoiding growth of pathogens and spoilage microorganisms (Rejano, 2010). After being submitted to one of those treatments, table olives are preserved in brine according to their specific characteristics, or in dry salt under modified atmosphere, upon addition of preservative or acidifying agent(s) or after pasteurization toward extended shelf-life (Portilha-Cunha *et al.*, 2020).

The submersion of table olives in salt is an important step for food preservation and for increase in fermentation rate (by stimulating diffusion of different compounds out of the cells), but also for taste and texture improvement (Mateus *et al.*, 2016). While fermentable substrates diffuse out of the olive flesh into the fermentation liquid, the salts diffuse into the olives, thus contributing to a final high salt level - which can impair the health image of this fermented food. Although the World Health Organization (WHO) recommends an intake of less than 5 g/day/person of salt, the salt consumption in Portugal is above 10 g/day/person. Hence, studies have looked for a reduction of salt in table olive brines (Mateus *et al.*, 2016; Saúde *et al.*, 2017).

Cobrançosa variety is subjected to natural fermentation - and is obtained before complete maturation, with variable colour, including pink and brownish shades; hence, this variety is considered mixed. When harvested during the maturation process with

the required size, table olives are considered green. The fruit size and shape are influenced by factors such as water availability, temperature, or even genetic differences; but fruits to be considered for table olive production must be above 4 g unit weight, and with a higher pulp content than seed. Moreover, shape, texture, colour, and flavour are important for consumer acceptance of the final product (Prata, 2015). The fermentation process starts spontaneously, and is strongly influenced by the olive cultivar itself, its indigenous microbiota, and such methodological factors as fermentation temperature and salt concentration of brines (Cosmai *et al.*, 2018); table olives harvested from higher and exterior levels of the tree tend to be more mature (Prata, 2015). Since *Cobrançosa* table olives only undergo a medium maturation process, there is a significant level of remaining phenolic compounds, responsible for their typical bitter flavour (Albuquerque *et al.*, 2019). Although the microbiological changes and physicochemical maturation of olives and brines, during processing, have been thoroughly investigated in Spain, Greece and Italy, the information about *Cobrançosa* Portuguese cultivar is scarce.

When searching for the terms ("Portugal" OR "Portuguese" OR "*Cobrançosa*") AND "table olives" thorough literature (Title, Abstract and Keywords) on databases such as SCOPUS, Science Direct and PubMed, a total of 48 documents can be found; however, only 10 are research articles related to *Cobrançosa* table olives, concerning characteristics such as microbiological (Pires-Cabral *et al.*, 2018), physicochemical and nutritional (Malheiro *et al.*, 2012; Pires-Cabral *et al.*, 2018; Prata *et al.*, 2019; Rodrigues *et al.*, 2022), sensory (Malheiro *et al.*, 2012; Rodrigues *et al.*, 2019), phenolic composition and antioxidant activity (Malheiro *et al.*, 2014; Malheiro *et al.*, 2011b; Rodrigues *et al.*, 2019; Rodrigues *et al.*, 2022; Seabra *et al.*, 2010; Sousa *et al.*, 2014), or even volatile profile (Malheiro *et al.*, 2011a) of the drupe; even so, none of these studies discuss the probiotic potential of lactic acid bacteria, neither the addition of a starter culture to this type of cultivar.

3. Relevance of a probiotic starter culture

Despite its economic significance, table olive fermentation is still mainly craft-based and empirically driven by its autochthonous microbiota – so it depends on various intrinsic and extrinsic factors, and results in a spontaneous process, that leads to unpredictable and variable quality of the final product, besides the possibility of growth of undesirable strains. Diverse microbial populations are involved in olive fermentation, mainly lactic acid bacteria (LAB) and yeasts; however, LAB are the main

responsible for brine acidification, due to lactic acid production - which leads to a decrease of pH and higher microbiological stability in the final product, as well as an extended shelf life. LAB have been extensively applied to traditional and industrial fermentations of dairy products such as yogurt or cheese, fermented cereals such as sourdough, and plant-based fermented products such as sauerkraut, kimchi, and olives. They are known for such features as adverse condition resistance, biopreservation, off-flavour elimination, antinutritional factor removal, antifungal activity, anti-mycotoxigenic and mycotoxin-binding ability, and proteolytic action; hence, they are often candidates for potentially probiotic microorganisms, and suitable as starter culture in the production of fermented foods (Bonatsou *et al.*, 2017; Kavitake *et al.*, 2018; Sedó Molina *et al.*, 2022). These usually consist of *Lactiplantibacillus plantarum* and *Lactiplantibacillus pentosus* or both (Cosmai *et al.*, 2018).

Over the years, it became important to define starter cultures by isolating native microflora from olive fermentations, and studying their technological characteristics and their probiotic potential. Inoculation with those starter cultures may contribute to the reduction of spoilage risk, enable controlled, optimized, standardized and shorter fermentation processes, and achieving a final product of higher quality, with improved organoleptic, nutritional, and functional properties. Nevertheless, maintenance and viability of starter cultures in fermented foods is still a great challenge in industrial processes (Bonatsou *et al.*, 2017; Kavitake *et al.*, 2018).

From starter cultures, two types can be pinpointed: selected (SSC) and natural starter cultures (NSC). SSC, commonly used at industrial level, are simpler, made up of a low, defined number of species and strains (sometimes monocultures) chosen for their technological and growth abilities - which makes them easily dominant over the natural microflora, when added to high concentrations. On the other hand, NSC are complex and undefined cultures, made of an ill-defined number of species and strains. When added to matrices, starter and non-starter strains coexist at equilibrium; although providing higher biodiversity, they are harder to standardize and reproduce (Campus *et al.*, 2018).

Appropriate microbial starters should exhibit some specific biotechnological and safety traits: easy and rapid adaptation to the brine environment (e.g., temperature, pH, and phenolic profile); rapid dominance against indigenous microbiota; rapid brine pH reduction (e.g. by producing organic acids); high enzymatic activity to enhance sensory characteristics of the final product; and rapid degradation of oleuropein (for

example, via enzymatic activity of ß-glucosidase and esterase), thus making the product edible (Anagnostopoulos and Tsaltas, 2022).

4. Previous work and main goals

Previous work for starter culture characterization and assessment of *in vitro* probiotic characteristics (see **Figure 1**) has been performed: isolation and identification of *Cobrançosa* native microflora during fermentation processes like *Lactiplantibacillus* (*Lpb.*) *pentosus* i106 (Reis *et al.*, 2022), selection for starters based on the best combination of technological properties (Rocha *et al.*, 2022), gastrointestinal performance, and safety for human consumption (Coimbra-Gomes *et al.*, 2022a), and probiotic characteristics (Coimbra-Gomes *et al.*, 2022b).

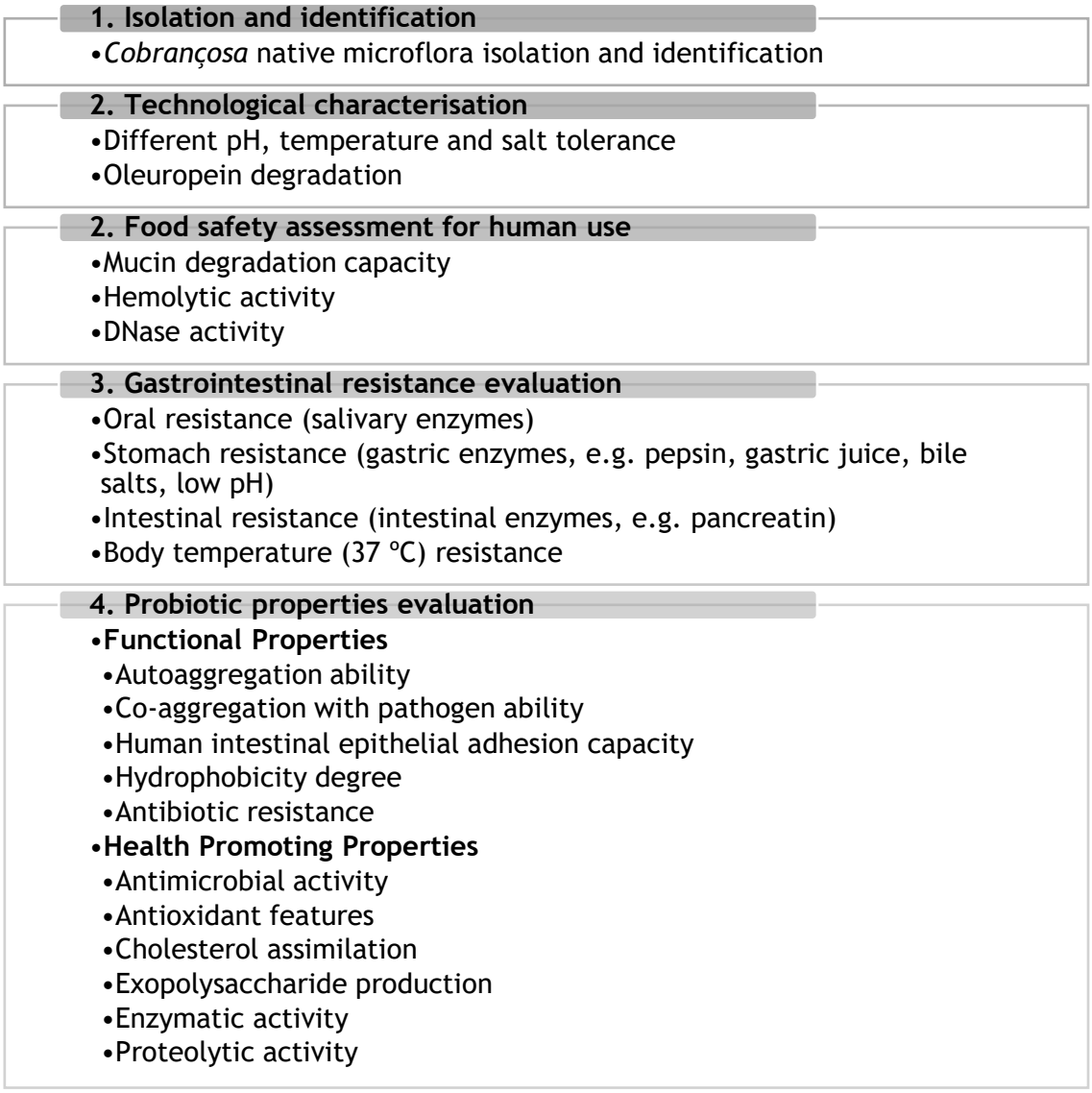

Figure 1. Steps for probiotic microorganism characterization and starter culture selection.

The main goal of this study was to test and validate, at "bench scale", the best LAB candidate in terms of both starter and probiotic properties - as a monoculture (SSC) that should be added to olives, depending on the time and stage of the process of *Cobrançosa* table olives. By defining a microbial starter culture, the producer will be able to optimize its growth conditions throughout table olive processing, and achieve a final product of higher quality - with improved functional properties beyond their pre-existing sensory and nutritional features, and thus susceptible to reach a premium price in the modern food market. By proving its probiotic potential, it is possible to intensify their intrinsic cultural value and enhance their status of food (Pires-Cabral *et al.*, 2018). For this purpose, this project comprised a study of microbial (LAB, yeasts and *Enterobacteriaceae* populations) throughout *Cobrançosa* table olive fermentation under different conditions, as well as brine physicochemical monitoring (salt, pH, titratable acidity, and phenolic compound concentration), complemented by analysis of the impact on table olive nutritional and sensory properties.

II. MATERIALS AND METHODS

1. Table olive fermentation assays

1.1. Table olives samples

Olives of cultivar *Cobrançosa* were provided by a producer (Trás-os-Montes, Northeast of Portugal), who use the natural fermentation method. For the experiments, the company supplied our laboratory with 5 L vessels of 4 kg of olives with water (plan A, ten vessels; plan B, six vessels), and with brine (5% salt) (plan C, four vessels). While table olives for plan A and B were from the same harvest of 2020/2021, tables olives for plan C were from an earlier harvest, 2019/2020 – see **Figure A1**; **Appendix I**. All vessels were covered and stored at room temperature.

1.2. Bacterial strains and growth conditions

Lactiplantibacillus pentosus strain i106, previously collected from *Cobrançosa* table olives and brines (Reis *et al.*, 2022), and selected for its probiotic potential (Coimbra-Gomes *et al.*, 2022a), was used in this study. Prior to the experiments, and following the methodology reported by Blana *et al.* (2013), such strain was revived from a stock culture (in 15% glycerol) at -80ºC, subcultured in 10 mL of de Man Rogosa Sharpe (MRS) broth (VWR Chemicals, Leuven, Belgium) with 0 or 3% of NaCl (w/v) and incubated at 37 ºC for 8-10 hours. After incubation, an aliquot was transferred into 90 mL of fresh MRS broth with 0, 3 or 5% of of NaCl (w/v) (depending on the trial) to obtain an initial OD_{600nm} of ca. 0.1. After overnight incubation at 30 ºC, a "working medium", prepared with mineral water, 2% of peptone from meat (VWR), 2% of glucose (Sigma-Aldrich, St. Louis, MO, USA) and 0, 3, 5 or 7% of NaCl (w/v) (depending on the trial to be inoculated), was inoculated with an aliquot of the overnight culture to give an initial OD_{600nm} of ca. 0.2, and incubated for 5-6 hours at 37 ºC. Finally, the trial vessels were inoculated with an initial OD_{600nm} of ca. 0.1. Initial concentration of LAB should reach 10^8 CFU/mL in each vessel. Gradual increases in NaCl concentration along inoculum preparation are essential to allow adaptation of the strain to the saline environment prevailing in brine (Panagou *et al.*, 2008).

1.3. Experimental design

Table olives fermentation from two different designs (A and B) were monitored over 35 weeks (245 days), and table olives from design C monitored over 10 weeks (65

days), according to **Figure 2**. Plan A evaluated the addition of strain *Lpb. pentosus* i106 to 5 independent trials: table olive water with no salt (trial A0) - obtained right after one week of harvest by the producer, and brines with 3 (trial A3), 5 (trial A5) and 7% (trial A7) of commercial salt; plan B tested the addition of LAB to the brine at 5 (trial B5) and 7% (trial B7) of salt, during the salting process of fermentation - i.e. obtained after sweetening *in loco* by the producer); and plan C assessed the LAB addition in ready-to-eat table olives (trial C5) - i.e. obtained after sweetening and salting *in loco* by the producer. Although only one trial has been inoculated in each inoculation moment, the salt was added to all the vessels. It is also important to mention that in plan A, no water renewal had been originally planned; however, at the time of inoculation with 3% of salt, it was found that the waters were not translucent, most likely pointing at diffusion of pulp compounds into the water. As suggested by the producer, a new water renewal was carried out in trials A3, A5 and A7 - which obviously reflected in the actual results. For all the plans, a control trial was also monitored (control trials AC, BC, and CC). The only difference between control and experimental trials was the fact that there was no addition of the probiotic LAB strain. All trials were run in duplicate.

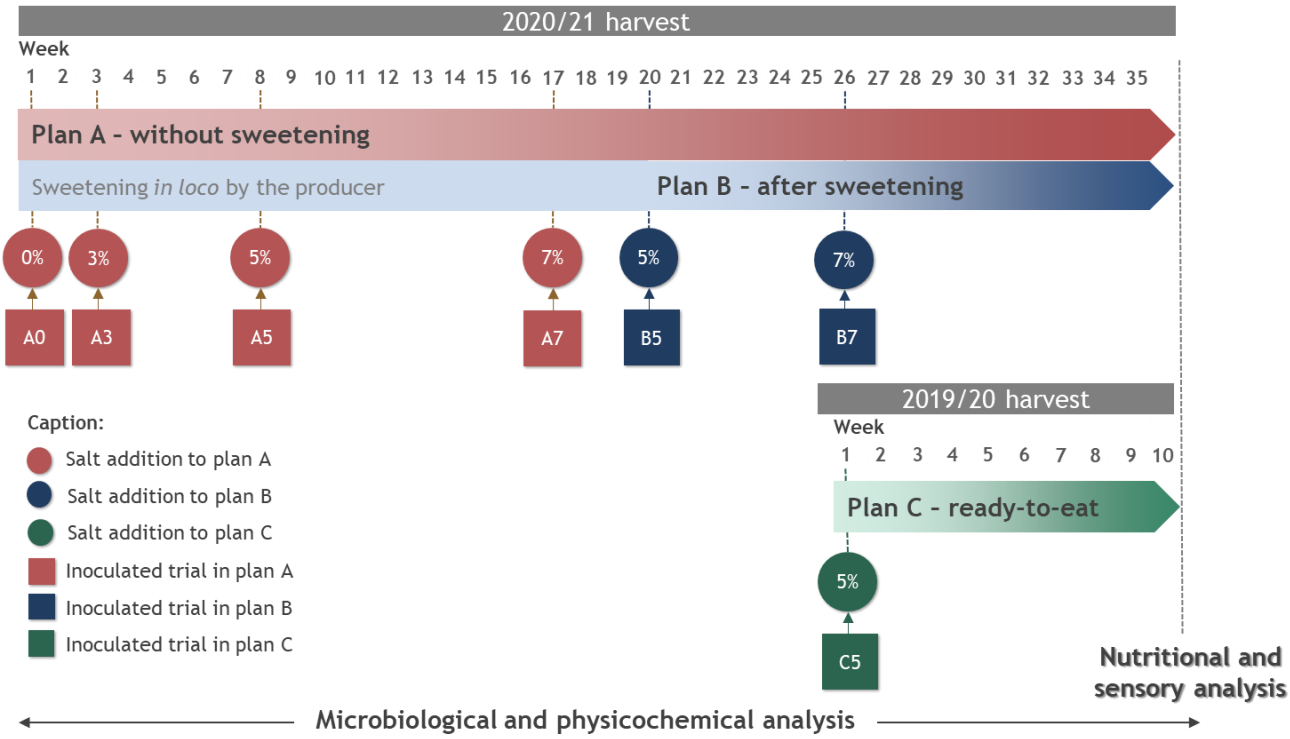

Figure 2. Scheme of the experimental design showing the moments of *Lpb. pentosus* i106 inoculation in the different strategies (plan A, B, and C) and salt addition, to vessels with *Cobrançosa* olives.

1.4. Microbiological analysis

In an aseptic environment, around 20 table olives and sufficient brine to cover them were randomly collected from each vessel with sterilized utensils. Twelve pitted olives (around 40 g) were placed in a sterile bag with side filter, together with 100 ml of saline solution (0.85%), with the aid of sterile material, and rubbed by a Stomacher Star Blender LB 400 (VWR, Milano, Italy) for 60 seconds; the filtered liquid was then poured into a sterile Falcon flask. Both the filtered liquid and brine were directly used for further dilution and plated. The methods of Abriouel *et al.* (2011) were followed for enumeration of yeasts and LAB in the samples. Enumeration of LAB and yeasts were monitored by spread plating table olives and brine samples in MRS agar with 0.4 g/L sodium azide (VWR) and Rose Bengal with 0.1 g/L Chloramphenicol (RBC) agar (VWR), respectively. Enumeration of *Enterobacteriaceae* were monitored by pour plating on Violet Red Bile Glucose (VRBGA) agar (VWR). Results were expressed as log CFU/mL for brine and as log CFU/g for olive samples (see **Appendix II** for complete results).

1.5. Physicochemical analysis

1.1.1. Salt content, pH and titratable acidity

Physicochemical analysis encompassed salinity, pH and titratable acidity of table olive brines. For measurement of brine salinity (expressed as percentage, w/v), a portable refractometer ATC (SSA-0010, YIERYI, Shzen Zhen, China) was used. The pH was determined by potentiometry, using a MU 6100 L pHenomenal® pH meter (VWR). The determination of Total Titratable Acidity (TTA) was performed according to Fernandez-Diez *et al.* (1985) and expressed as percentage of lactic acid (w/v). All analysis were performed in duplicate after calibration of the equipment. Although analysis were performed in several days of experiment, only the most relevant were considered as results (see **Appendix III** for complete results).

1.1.2. Phenolic compounds analysis

The concentration of phenolic components of table olive brines (i.e., oleuropein, verbascoside, hydroxytyrosol and tyrosol (see **Figure A5** and **Figure A6**; **Appendix IV**) was obtained by HPLC (High-performance liquid chromatography), after the samples were filtered through a 0.2 µm filter (VWR). All assays were performed in duplicate for each sample. HPLC analysis were performed with a Waters (Milford, MA, USA)

Alliance HPLC system (model 2695), equipped with photodiode array detector (model 2998). The chromatographic separations were performed by a reverse phase HPLC, using a C18 Cortecs column (4.6 mm x150 mm, 2.7 µm) (Waters) coupled to a C18 Cortecs VanGuard (3.9 x 5 mm, 2.7 µm), maintained at 35 ºC. Ultrapure water with 0.02% TFA (VWR Chemicals) (A) and pure acetonitrile (VWR Chemicals) (B) were used as mobile phases. Analytes were separated using the following gradient: 0 min, 5% B; 0-3 min, 20% B; 3-4 min, 40% B; 4-10 min, 80% B; 10-11 min, 80% B; 11-13 min; and the column was equilibrated with 95% A for 4 min between each run. The mobile phase flow was 1 mL/min, and the column temperature was 35 °C. Samples were preserved at 4 ºC. Calibration standards, oleuropein (≥ 80%), hydroxytyrosol (≥ 98%), tyrosol (≥ 99.5%), verbascoside (86%), and syringic acid (95%) (Sigma-Aldrich), were used for quantitative measurement of sample compounds. Selection of the aforementioned polyphenols was based on their relative abundance and importance for table olive brines. Calibration curves were built with different (known) concentrations (**Table A11**; **Appendix IV**); for oleuropein, hydroxytyrosol, and tyrosol (from 150 to 1000 mg/L), verbascoside (from 150 to 500 mg/L), and syringic acid (ranging from 2 to 100 mg/L), standard solutions were prepared in methanol/H_2O (20/80). All solutions were filtered through 0.45 µm syringe filters (VWR). Detection and quantification limits were determined for each curve, at 240 nm and 330 nm (maximum absorbance wavelength for oleuropein and verbascoside, respectively); no significant variation occurred of absorbance at wavelength other than 280 nm (maximum absorbance wavelength for hydroxytyrosol and tyrosol), thus justifying operation only at that wavelength. The injection volume for standards and sample extracts was 20 µL; for each run and elution profile, detection was at 280 nm using photodiode detectors. Oleuropein and the other phenolic compounds in the analysed sample were identified by matching retention time of a characteristic peak in the extract/sample chromatogram with the peak of a known standard compound. Concentrations of compounds were expressed in mg/L and as a mean of two determinations. Complete results and examples of chromatograms for some trials can be accessed in **Appendix IV**.

1.6. Nutritional analysis

Table olive nutritional profile was determined at the end of the microbiological and physicochemical experiment. Before the analysis, the table olive pits were removed, and the pulp was milled in a Knife Mill Grindomix GM 200 (Retsch) at 8000 rpm for 20 seconds. A table olive paste was obtained and stored in individual flasks. All procedures that required sample weighing were done using an Analytical Balance

(KERN AES). All results obtained by the following procedures were represented by mean ± standard deviation of two replicates.

1.6.1. Water Content

Water content was determined via the AOAC method 950.46 (Horwitz, 2000). Using a Moisture Analyser DBS (KERN), approximately 3 g of sample was weighed and dried at 102 ºC until constant weight. The difference in weight allowed the calculation of the water content, in percentage.

1.6.2. Ash Content

Ash content was determined via the AOAC method 945.05 (Horwitz, 2000); 5 g of sample was weighed into a porcelain crucible, and placed in a 48000 Muffle Furnace (Thermolyne) operated at 525ºC. The procedure lasted for at least 24 h. The procedure was complete when the sample turns completely white.

1.6.3. Total Lipids

Total lipids were extracted with recycled petroleum ether, according to AOAC method 920.39 - Soxhlet extraction (Horwitz, 2000). Before extraction, around 5 g of sample was weighed. Flat bottom flask, where the extraction takes place, was previously dried and cooled in a desiccator. An equal amount of anhydrous sodium sulphate (Merck, Germany) was added and mixed with the sample to remove water. The mixture was transferred to a cellulose test tube, and put into a heat reflux extractor connected to a condenser. After at least 6 h of extraction, both ether and lipids reside in the flat bottom flask. Most ether was recycled by distillation, and the rest of the mixture dried at 100ºC was repeatedly weighed until constant weight.

1.6.4. Crude Protein

Protein content was determined via the AOAC method 2001.11 (Kjeldahl method) (Horwitz, 2000). Approximately 1 g of sample is put in a digestion tube and mounted on a Speed Digester K-425 (BÜCHI). The sample is then digested in H_2SO_4, to convert the protein nitrogen to $(NH_4)_2SO_4$ at a boiling point elevated by addition of Kjeldahl tablets (Merck): K_2SO_4 with a Cu catalyst to was used to enhance the reaction rate. During the 2 h-digestion, 20 mL of 0.2 N NaOH was measured. The $(NH_4)_2SO_4$ formed from digestion evaporated as ammonia, liberated by alkaline steam distillation using a Distillation Unit K-350 (BÜCHI). The ammonia was quantified titrimetrically with 0.2 N H_2SO_4. The analyte is called "crude" protein because the method determines N, a component of all proteins. In addition, any N from sources other than true protein is

also determined with this method. The amount of protein is then calculated by multiplying the %N by 6.25, since proteins contain 16% N or average.

1.6.5. Total Dietary Fibre

Total fibre content analysis followed AOAC Method 985.29 (Horwitz, 2000), with some modifications - since the table olive fat content exceeds 15%, thus requiring prior fat extraction (Soxhlet extraction).

1.6.6. Carbohydrates

Total carbohydrates are calculated considering the following formula:

$$Carbohydrates\ (\%) = 100 - (Water + Fat + Protein + Ash + Fibre)\ contents$$

1.6.7. Salt content

Sodium chloride content of the table olives pulp was determined by titration with $AgNO_3$ (Mohr method), according to the method of Fernandez-Diez *et al.* (1985).

1.6.8. Energetic value

Similarly to Malheiro *et al.* (2012), the energetic value was expressed as kcal/100 g of fresh fruit, using the Atwater classical factors for protein (4), fat (9), carbohydrates (4) and dietary fibre (2), according to the formula:

$$Energy\ (kcal) = 4\ (g\ protein + g\ carbohydrates) + 9\ (g\ fat) + 2\ (g\ fibre)$$

1.7. Sensory analysis

Following Anagnostopoulos *et al.* (2020a), table olive samples from all experimental plans were evaluated at the end of the process by 12 panel members of Casa Agrícola Quinta da Cruzinha, Lda. (Mosteiró, Torre D. Chama, Mirandela) - 5 males and 7 females, aged from 40 to 60 years old, according to Regulation COI/OT/MO No 1/Rev.3 (IOC, 2021). Texture, flavour, saltiness, acidity, bitterness, off flavours (abnormal flavours), and overall acceptance were assessed - and rated according to a 10 cm scale line, as shown in the small questionnaire presented in **Appendix V.** Further characteristics considered relevant by any panellist member could also be added in the questionnaire. Results were presented in radar charts, considering the means, standard deviations, and Robust Coefficient of Variation (CVr), as per the above Regulation (see **Appendix V** for complete results).

2. Statistical analysis

All graphics and calculations of means and standard deviations were executed in Excel (Microsoft® Office 2021). A one-way analysis of variance (ANOVA), followed by Tuckey Post-Hoc means multiple comparisons, was performed for physicochemical and nutritional characteristics, with the aid of IBM SPSS 27.0 software (IBM, Armonk, NY, USA) – at 5% level of significance. Letters in mean comparisons were ascribed, as proposed by Piepho (2018). The same software was used for mean multiple comparison of overall eating quality results.

III. RESULTS

1. Microbiology monitoring

1.1. Plan A - Olive fermentation without sweetening

Figure 3, Figure 4 and **Figure 5** represent the microbial changes of LAB, yeasts and *Enterobacteriaceae*, respectively, present in brine and in table olives during *Cobrançosa* table olive fermentation in plan A (see **Appendix II** for complete results).

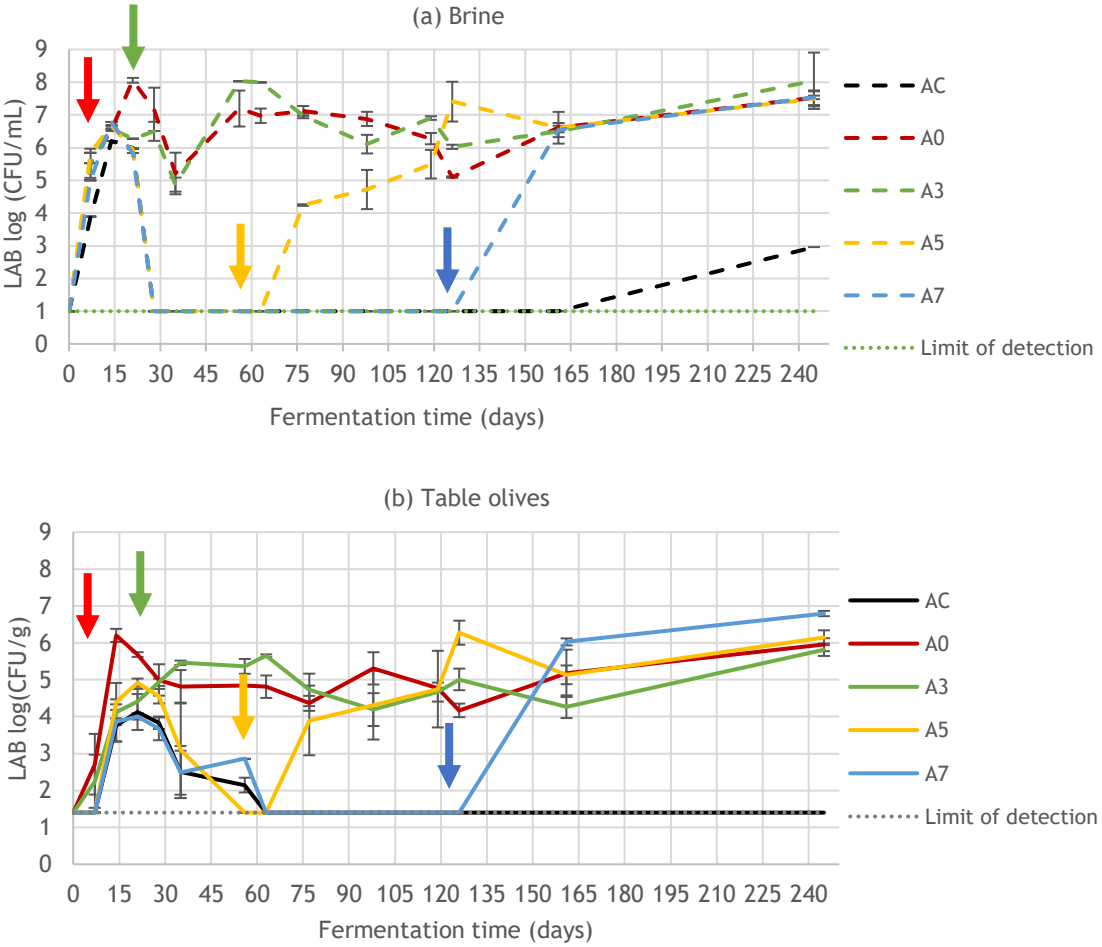

Figure 3. Plan A: Lactic acid bacteria microbial monitoring in (a) brine and (b) table olives. Arrows correspond to the moment of inoculation with *Lpb. pentosus* i106 to the experimental trial, and the corresponding salt addition to all vessels.

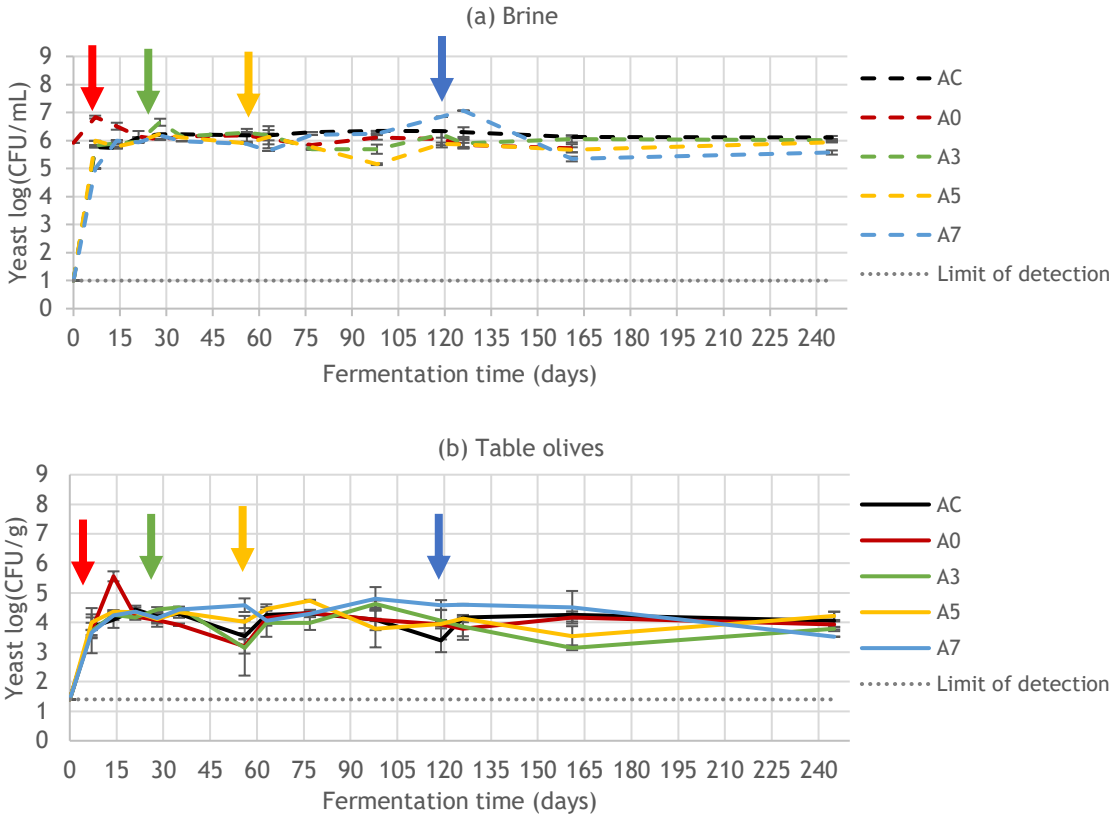

Figure 4. Plan A: Yeasts microbial monitoring in (a) brine and (b) table olives. Arrows correspond to the moment of inoculation with *Lpb. pentosus* i106 to the experimental trial, and the corresponding salt addition to all vessels.

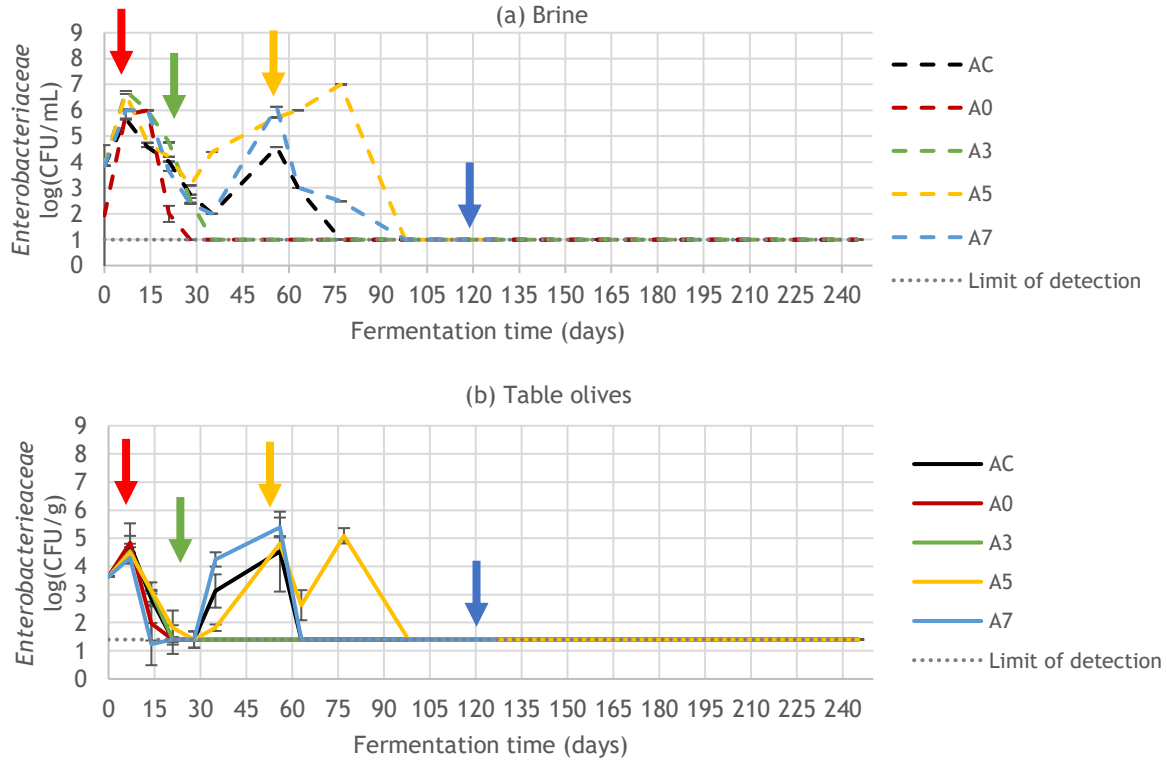

Figure 5. Plan A: *Enterobacteriaceae* microbial monitoring in (a) brine and (b) table olives. Arrows correspond to the moment of inoculation with *Lpb. pentosus* i106 to the experimental trial, and the corresponding salt addition to all vessels.

LAB population (**Figure 3**) maintained high, at 7 log CFU/mL in brine and 5 log CFU/g in table olives, for the batches where the probiotic strain was added at early stages (trials A0 and A3). In batches where strain i106 was added later (trials A5 and A7), the LAB population was initially high, followed by a significant decrease; after addition of the probiotic strain, LAB populations have recovered to 7 log CFU/mL in brine and 5-6 log CFU/g in table olives. Regarding the control batches (trial AC), after some initial growth, LAB populations have decreased and remained below the detection limit; however, by the end of the experiment, some LAB growth was observed in brine. Regarding yeasts population (**Figure 4**), and despite an initial increase, it has stabilized at 5 log CFU/mL and 4 log CFU/g in brine and table olives, respectively. As expected, *Enterobacteriaceae* population (**Figure 5**) decreased with time, either in brine or table olives, until reach values below the detection limit.

1.2. Plan B - Olive fermentation after previous sweetening

Figure 6 and **Figure 7** present LAB and yeasts populations, respectively, in plan B. No *Enterobacteriaceae* counts were recorded.

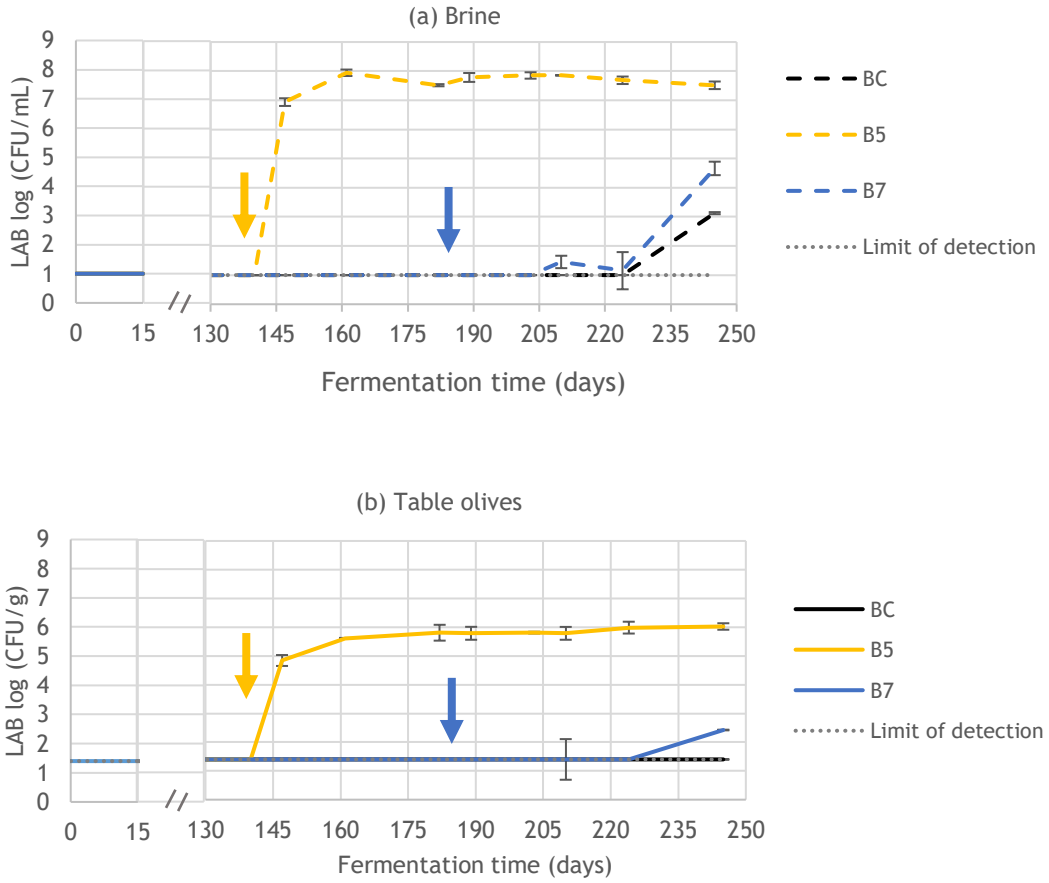

Figure 6. Plan B: Lactic acid bacteria microbial monitoring in (a) brine and (b) table olives. Arrows correspond to the moment of inoculation with *Lpb. pentosus* i106 to the experimental trial, and the corresponding salt addition to all vessels.

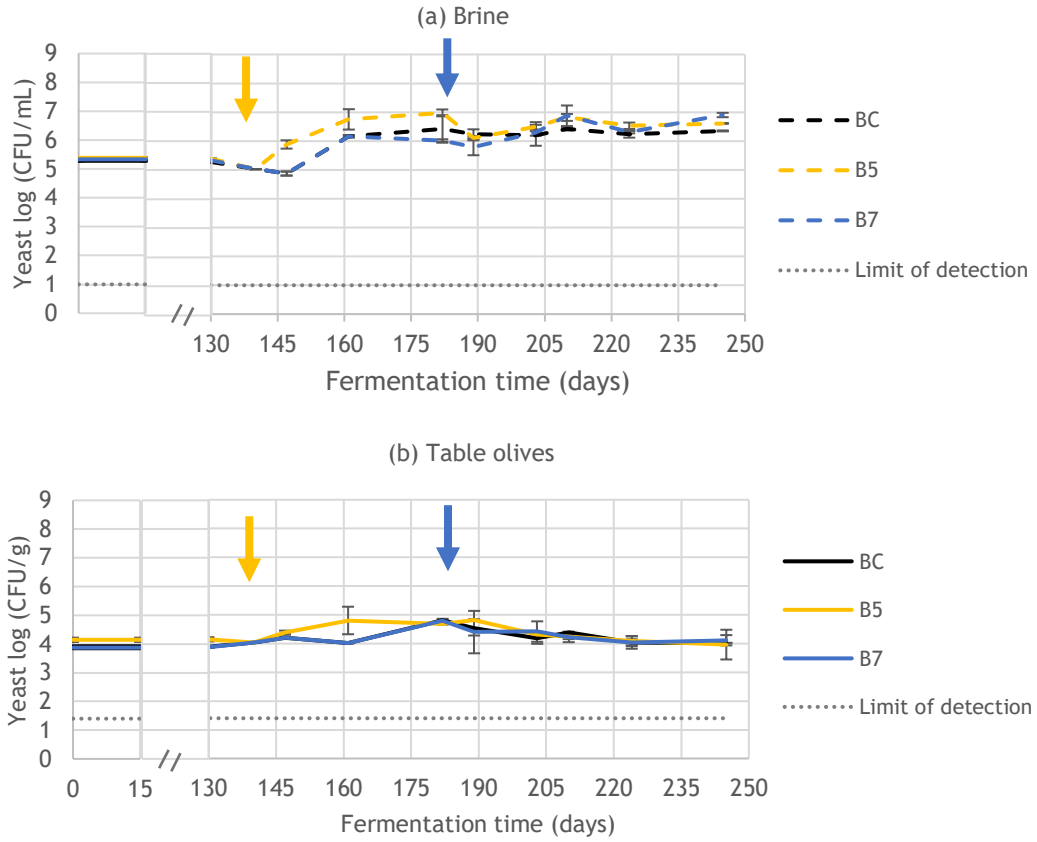

Figure 7. Plan B: Yeasts microbial monitoring in (a) brine and (b) table olives. Arrows correspond to the moment of inoculation with *Lpb. pentosus* i106 to the experimental trial, and the corresponding salt addition to all vessels.

Significant differences were recorded between trials regarding LAB populations (**Figure 6**): trial AC presented LAB counts below the detection limit during the whole experiment, except at the end in brine, when some LAB growth was verified reaching ca. 3 log CFU/mL; trial A5 showed a LAB growth to 7-8 log CFU/mL in brine and 5-6 log CFU/g in table olives 7 days after addition of the probiotic, and remained constant until the end of the experiment; finally, trial A7 presented a slower growth, showing some LAB growth after 30 days, and only reaching 4 log CFU/mL in brine and 2 log CFU/g in table olives. Regarding yeast populations (**Figure 7**), no significant differences were registered during the experiment in the fruit (4 to 5 log CFU/g), neither between trials; in brine, a growth of 1-2 log CFU/mL was observed after 140 days, which stabilized at 6 to 7 log CFU/mL, also with no significant differences between trials.

1.3. Plan C - Fermentation with ready-to-eat olives

Considering plan C – and similarly to plan B, none of the trials presented *Enterobacteriaceae* populations. LAB and yeasts populations are presented in **Figure 8** and **Figure 9**, respectively.

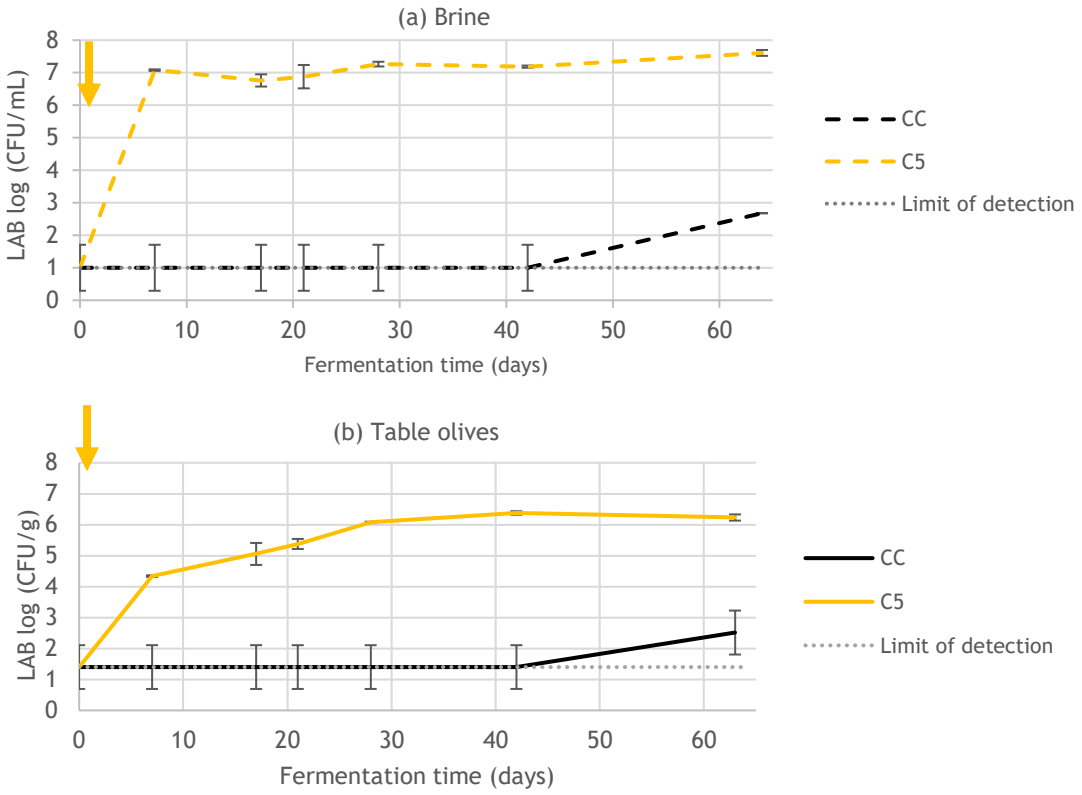

Figure 8. Plan C: Lactic acid bacteria microbial monitoring in (a) brine and (b) table olives. Arrows correspond to the moment of inoculation with *Lpb. pentosus* i106 to the experimental trial, and the corresponding salt addition to all vessels.

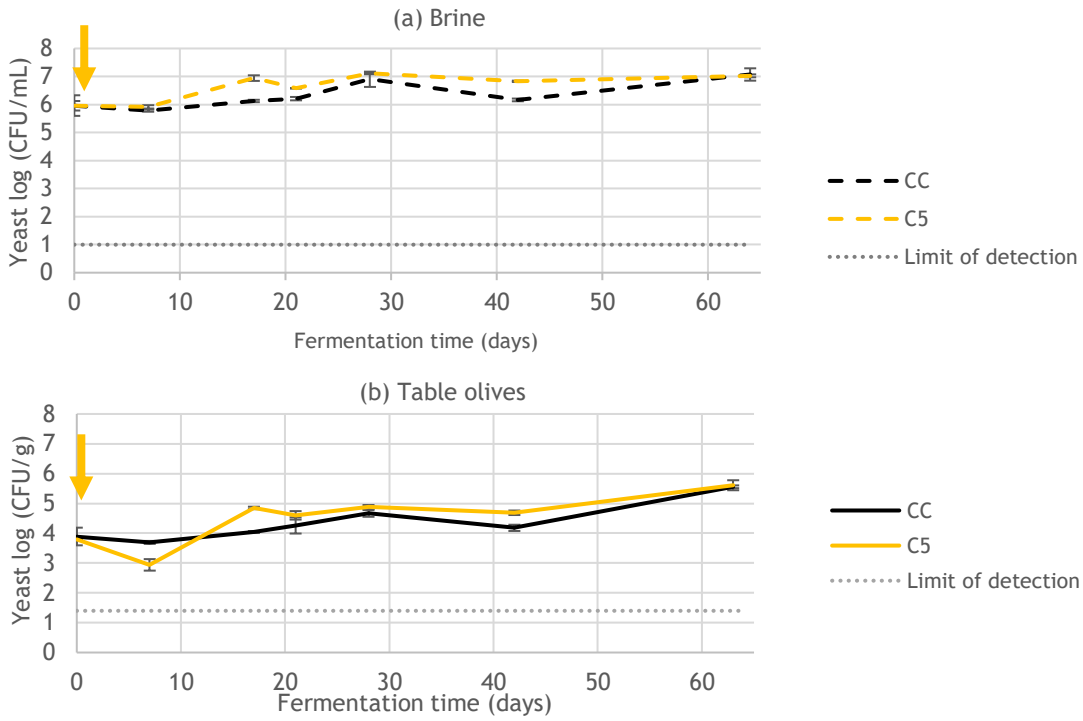

Figure 9. Plan C: Yeasts microbial monitoring in (a) brine and (b) table olives. Arrows correspond to the moment of inoculation with *Lpb. pentosus* i106 to the experimental trial, and the corresponding salt addition to all vessels.

A significant increase of LAB populations (**Figure 8**) was observed along the process after strain i106 addition, either in brine (up to 8 log CFU/mL) or table olives (up to 6.5 log CFU/g). Regarding the control trial (CC), after 40 days of fermentation, LAB populations also started to grow, reaching 1 log CFU/mL in brine and 2 log CFU/g in table olives. Concerning yeast populations (**Figure 9**), no significant difference was recorded in brine among trials, neither along the experiment; on the other hand, in table olives there was a small growth (1.5 log CFU/g) when comparing the end with the beginning of the experiment, yet no differences were found among trials.

2. Physicochemical monitoring

2.1. Salt content, pH and titratable acidity

Table 1, **Table 2** and **Table 3**, represent the physicochemical properties of plan A, B and C, respectively, concerning some significant times of fermentation - in the beginning of each experiment (time 0), one week after addition of each salt level and *Lpb. pentosus* i106 to each trial, and finally at the end of the experiment (complete results can be retrieved in **Appendix III**).

2.1.1. Plan A - Olive fermentation after previous sweetening

Salt content among trials did not differ significantly ($p>0.05$) for each time of experiment - only differing within each trial throughout the days, as the salt was added. Regarding pH values, all trials began at 5.2 and two patterns of variations can be detected afterwards. First, the trials where *Lpb. pentosus* i106 was added with low percentage of salt (A0 and A3) exhibited a significant decrease in pH (3.8 and 3.6, respectively) when compared to control (4.4) at early stages of fermentation, then they approximated the control (ca. 4.1) around 3 months, and maintained that level until the end (ca. 4.7). Second, for the trials where *Lpb. pentosus* i106 was added with medium/high percentage of salt (A5 and A7), the pH profile was similar between the two experiments and the control (ca. 4.1) until ca. 3 months; however, one also observed that, after the salt content reached the value of 5%, the pH tended to increase in trials A5 and A7 (up to 5.1) - while the others kept a stabilized value (ca. 4.7, as mentioned before). Acidity percentages started with ca. 0.16% for all trials, then reached a maximum value depending on the trial, followed by a reduction down to ca. 0.20%. Trials A5 and A7 exhibited a decrease in TTA at early stages of fermentation (of 0.10% by 28 days), when LAB was not detected; this behaviour was not found for control, neither for A0 and A3 - where LAB were detected - see **Table 1**.

2.1.2. Plan B - Olive fermentation after previous sweetening

The profiles for salt content, pH, and TTA were similar to those described for plan A. However, some differences in the values were found, considering that plan B started after the sweetening phase. The pH value at the beginning was slightly lower (4.17) than plan A and, the same happened at the end (4.2 for AC, and ca. 4.75 for trials A5 and A7). The TTA value at the beginning was almost one third that of plan A (0.05%), and consequently the same ratio occurred at the end (0.11% for AC and below 0.10% for trials A5 and A7) – see **Table 2**.

2.1.3. Plan C – Fermentation with ready-to-eat olives

During the process, after the addition of *Lpb. pentosus* i106 to the final product it was verified a decrease of the pH value (3.6) and an increase of TTA (0.29%) when compared to the control (4.04 and 0.14%, respectively, for pH and TTA). Anyway, as happened with plans A and B, at the end of the process, the pH and TTA tended to be different (4.46 and 0.07%), but closer to those of the control (i.e. 4.40 and 0.10%) – see **Table 3**.

Table 1. Physicochemical properties (salt content, pH and total titratable acidity) of brine at significant times of fermentation of plan A.

	Trial	Fermentation time (days)						p-value
		0	14	28	63	126	245	
Salt (%)	AC	0.00 ± 0.00^{A}	0.00 ± 0.00^{A}	2.90 ± 0.14^{aB}	5.00 ± 0.00^{C}	6.70 ± 0.28^{D}	5.50 ± 0.00^{aC}	< 0.001
	A0	0.00 ± 0.00^{A}	0.00 ± 0.00^{A}	3.80 ± 0.28^{bB}	5.00 ± 0.00^{C}	6.75 ± 0.35^{E}	5.80 ± 0.00^{abD}	< 0.001
	A3	0.00 ± 0.00^{A}	0.00 ± 0.00^{A}	3.00 ± 0.00^{aB}	5.00 ± 0.00^{C}	6.25 ± 0.35^{E}	5.65 ± 0.07^{abD}	< 0.001
	A5	0.00 ± 0.00^{A}	0.00 ± 0.00^{A}	2.85 ± 0.07^{aB}	5.00 ± 0.00^{C}	6.00 ± 0.00^{D}	5.95 ± 0.21^{bD}	< 0.001
	A7	0.00 ± 0.00^{A}	0.00 ± 0.00^{A}	3.00 ± 0.00^{aB}	4.90 ± 0.14^{C}	6.80 ± 0.28^{E}	5.50 ± 0.00^{aD}	< 0.001
p-value		1.000	1.000	0.006	0.486	0.124	0.023	
pH	AC	5.20 ± 0.00	4.56 ± 0.01	4.40 ± 0.03^{b}	4.44 ± 0.03	4.20 ± 0.07^{ab}	4.56 ± 0.06^{a}	0.082
	A0	5.20 ± 0.00^{E}	4.93 ± 0.01^{D}	3.83 ± 0.01^{aA}	3.95 ± 0.04^{A}	4.22 ± 0.06^{abB}	4.65 ± 0.13^{abC}	< 0.001
	A3	5.20 ± 0.00^{D}	4.62 ± 0.01^{C}	3.87 ± 0.11^{aAB}	3.61 ± 0.03^{A}	4.09 ± 0.09^{aB}	4.83 ± 0.09^{abcC}	< 0.001
	A5	5.20 ± 0.00^{C}	4.60 ± 0.05^{B}	4.40 ± 0.03^{bB}	4.54 ± 0.03^{B}	4.08 ± 0.02^{aA}	5.08 ± 0.16^{bcC}	< 0.001
	A7	5.20 ± 0.00^{B}	4.60 ± 0.01^{A}	4.42 ± 0.02^{bA}	4.60 ± 0.01^{A}	4.39 ± 0.08^{bA}	5.19 ± 0.14^{cB}	< 0.001
p-value		1.000	0.584	< 0.001	0.532	0.030	0.010	
TTA*	AC	0.15 ± 0.04^{A}	0.18 ± 0.01^{bA}	0.20 ± 0.02^{bA}	0.23 ± 0.03^{aA}	0.40 ± 0.06^{abB}	0.23 ± 0.01^{A}	0.006
	A0	0.16 ± 0.01^{A}	0.40 ± 0.00^{dB}	0.56 ± 0.01^{cC}	0.54 ± 0.03^{bBC}	0.46 ± 0.04^{bBC}	0.27 ± 0.04^{BC}	< 0.001
	A3	0.17 ± 0.02^{A}	0.18 ± 0.00^{bA}	0.19 ± 0.01^{bA}	0.59 ± 0.01^{bD}	0.42 ± 0.04^{abC}	0.19 ± 0.03^{B}	< 0.001
	A5	0.15 ± 0.01^{AB}	0.16 ± 0.01^{aAB}	0.11 ± 0.00^{aA}	0.20 ± 0.02^{aAB}	0.51 ± 0.01^{bC}	0.17 ± 0.03^{B}	< 0.001
	A7	0.18 ± 0.01^{AB}	0.20 ± 0.00^{cB}	0.09 ± 0.00^{aA}	0.18 ± 0.03^{aAB}	0.27 ± 0.03^{aB}	0.14 ± 0.01^{B}	0.004
p-value		0.665	< 0.001	< 0.001	< 0.001	0.015	0.090	

* expressed as lactic acid g/100 mL of brine.
Each value in the table represents the mean ± standard deviation of two independent trials.
$^{a-c}$ Means followed by different lowercase letters in each column differed significantly among trials for a given sampling time, and $^{A-E}$ means followed by different capital letter in each row differed significantly among sampling times for a given trial ($p<0.05$) by Tuckey Post-Hoc comparison.

Table 2. Physicochemical properties (salt content, pH and total titratable acidity) of brine at significant times of fermentation of plan B.

	Trial	Fermentation time (days)				
		0	140	182	245	p-value
Salt (%)	BC	4.35 ± 0.21^{B}	3.75 ± 0.07^{aA}	6.50 ± 0.00^{D}	5.90 ± 0.14^{C}	< 0.001
	B5	4.35 ± 0.21^{A}	4.25 ± 0.07^{bA}	6.20 ± 0.28^{B}	5.55 ± 0.07^{B}	0.001
	B7	4.35 ± 0.21^{B}	3.75 ± 0.07^{aA}	6.50 ± 0.00^{D}	5.60 ± 0.00^{C}	< 0.001
p-value		1.000	0.009	0.253	0.057	
pH	BC	4.17 ± 0.05^{B}	3.98 ± 0.02^{bA}	4.01 ± 0.01^{aA}	4.17 ± 0.04^{aB}	0.009
	B5	4.19 ± 0.01^{B}	3.76 ± 0.02^{aA}	4.24 ± 0.02^{bB}	4.83 ± 0.08^{bC}	< 0.001
	B7	4.18 ± 0.04^{C}	3.98 ± 0.02^{bA}	4.00 ± 0.05^{aAB}	4.71 ± 0.04^{bD}	< 0.001
p-value		0.859	0.003	0.019	0.003	
TTA*	BC	0.05 ± 0.00^{A}	0.21 ± 0.01^{C}	0.12 ± 0.01^{abB}	0.11 ± 0.01^{bB}	< 0.001
	B5	0.05 ± 0.00^{A}	0.16 ± 0.02^{B}	0.15 ± 0.01^{bB}	0.07 ± 0.01^{aA}	0.003
	B7	0.05 ± 0.00^{A}	0.14 ± 0.02^{B}	0.07 ± 0.02^{aAB}	0.07 ± 0.01^{aAB}	0.038
p-value		1.000	0.130	0.017	0.032	

* expressed as lactic acid g/100 mL of brine.
Each value in the table represents the mean ± standard deviation of two independent trials.
$^{a-b}$ Means followed by different lowercase letters in each column differed significantly among trials for a given sampling time, and $^{A-D}$ means followed by different capital letter in each row differed significantly among sampling times for a given trial ($p<0.05$) by Tuckey Post-Hoc comparison.

Table 3. Physicochemical properties (salt content, pH and total titratable acidity) of brine at significant times of fermentation of plan C.

	Trial	Fermentation time (days)			
		0	7	64	p-value
Salt (%)	CC	5.10 ± 0.14	5.75 ± 0.35	5.75 ± 0.21	0.104
	C5	5.00 ± 0.00^{A}	6.00 ± 0.00^{C}	5.75 ± 0.07^{B}	< 0.001
p-value		0.423	0.423	1.000	
pH	CC	4.03 ± 0.04^{A}	4.04 ± 0.01^{A}	4.40 ± 0.08^{B}	0.011
	C5	4.10 ± 0.03^{B}	3.58 ± 0.01^{A}	4.46 ± 0.09^{C}	0.001
p-value		0.192	< 0.001	0.598	
TTA*	CC	0.14 ± 0.03	0.14 ± 0.02	0.10 ± 0.03	0.228
	C5	0.14 ± 0.01^{B}	0.29 ± 0.01^{C}	0.07 ± 0.01^{A}	< 0.001
p-value		0.771	0.005	0.312	

* expressed as lactic acid g/100 mL of brine.
Each value in the table represents the mean ± standard deviation of two independent trials.
Means followed by different lowercase letters in each column differed significantly among trials for a given sampling time, and $^{A-C}$ means followed by different capital letter in each row differed significantly among sampling times for a given trial ($p<0.05$) by Tuckey Post-Hoc comparison.

2.2. Phenolic compounds monitoring

Figure 10, **Figure 11**, and **Figure 12** compile information on the concentration of some of the phenolic components - oleuropein, hydroxytyrosol, tyrosol and verbascoside, in table olive brines from plans A, B and C, respectively - regarding some of the experimental times of fermentation, including the initial time, one week after each inoculation and, final time; complete results can be accessed in **Appendix IV**.

2.2.1. Plan A - Olive fermentation without sweetening

Observing **Figure 10**, none of the trials differ from each other at the beginning of the experiment. One of the first things to notice is that tyrosol is the phenolic compound with the lowest concentration values - see **Figure 10(c)**, showing little increases with time, after two weeks to one month, reaching maxima between 200 and 300 mg/L in the final stages of the process. The trial A0 presented higher tyrosol concentrations than the other trials. Regarding oleuropein concentration, all trials, except A0, presented a similar behaviour throughout fermentation - see **Figure 10(a)**, showing a slight decrease in the beginning of the experiment (from around 300 to 50-180 mg/L), followed by a significant increase (reaching maxima from 300 to 600 mg/L) and a gradual decrease right after. Inoculated trials (except trial A0) presented oleuropein concentrations below the control trial (AC) most of the fermentation time. The trial A0, besides not presenting an initial decrease of its oleuropein content (as observed for the other trials) exhibited instead a significant increase (up to ca. 850 mg/L) - with higher values of oleuropein found throughout the whole experiment; towards higher initial concentrations, the reduction of oleuropein until the end of the experiment was more significant than in the remaining trials. Similarly to oleuropein, all trials exhibited analogous hydroxytyrosol concentration evolution throughout the fermentation process - see **Figure 10(b)**, increasing from values around 100 mg/L to a maximum of 1126 mg/mL after 126 days, but presenting a significant decrease afterwards. Once again, the higher values were associated to trial A0 during the whole experiment; however, at the end of the process, the control trial presented equivalent higher values when compared to the remaining trials. Moreover, it is possible to identify an apparent correlation between oleuropein content decrease with hydroxytyrosol increase throughout the experiment. Concerning verbascoside, the evolution comprised once more an initial increase, beginning at 200 mg/L until a maximum of 800 to 1000 mg/L, followed by a significant decrease - the higher, the larger salt percentage of the trial; the higher value corresponds to the control trial,

but, the maximum verbascoside concentration was obtained later than the previous phenolic compounds - see **Figure 10(d)**.

2.2.2. Plan B - Olive fermentation after previous sweetening

One of the major observations in plan B is that no tyrosol was detected (i.e. concentration was below the detection limit – see **Table A11**; **Appendix IV**). Observing **Figure 11** at the beginning of the experiment, no differences were found among trials. Oleuropein content - **Figure 11(a)**, only differed throughout fermentation in trials BC and B7; an initial increase was present until a maximum of approximately 140 and 160 mg/mL, respectively. Afterwards, while BC remained constant over time, B7 underwent a significant decrease, a few weeks after inoculation. Regarding the B5 oleuropein concentration remained low throughout time (ca. 50 mg/L). It is also important to notice that inoculated trials reached final oleuropein concentrations lower than the control trial. The behaviour observed for hydroxytyrosol concentration - **Figure 11(b),** shows that a significant increase existed in all trials, followed by a stabilization until the end of the experiment. A higher content was reported in B7, reaching, approximately 250 mg/L; while BC stayed at ca. 150 mg/L level; B5 reported values very close the detection limit. Verbascoside concentration - **Figure 11(c)**, did also increase with time in all trials, from values around 50 to around 450 mg/L for BC and B7, respectively, and to ca. 300 mg/L for B5. Considering now all phenolic compounds, it is possible to verify that the overall concentration of each compound is much lower than the concentration values found for plan A.

2.2.3. Plan C – Fermentation with ready-to-eat olives

Finally, tyrosol was also not found in plan C. Regarding oleuropein content - **Figure 12(a)**, it remained constant throughout the experiment in both trials (CC and C5) in concentrations of c.a. 100-150 and 50-100 mg/L, respectively; besides, one always noticed slightly lower values for the trial C5. According to **Figure 12(b),** no significant differences were found for hydroxytyrosol content neither through time nor within trials; nevertheless, and like observed in the previous plans, hydroxytyrosol concentration shows a little increase until a maximum was reached (from 200 to 400 mg/L), and decreased afterwards to values from 200 to 300 mg/L, higher for the inoculated trial. Unlike oleuropein content evolution, verbascoside concentration - **Figure 12(c)**, started increasing at the very beginning of the experiment from, ca. 50 mg/L, to 500 and 250 mg/L, for CC and C5, respectively; and remained constant afterwards.

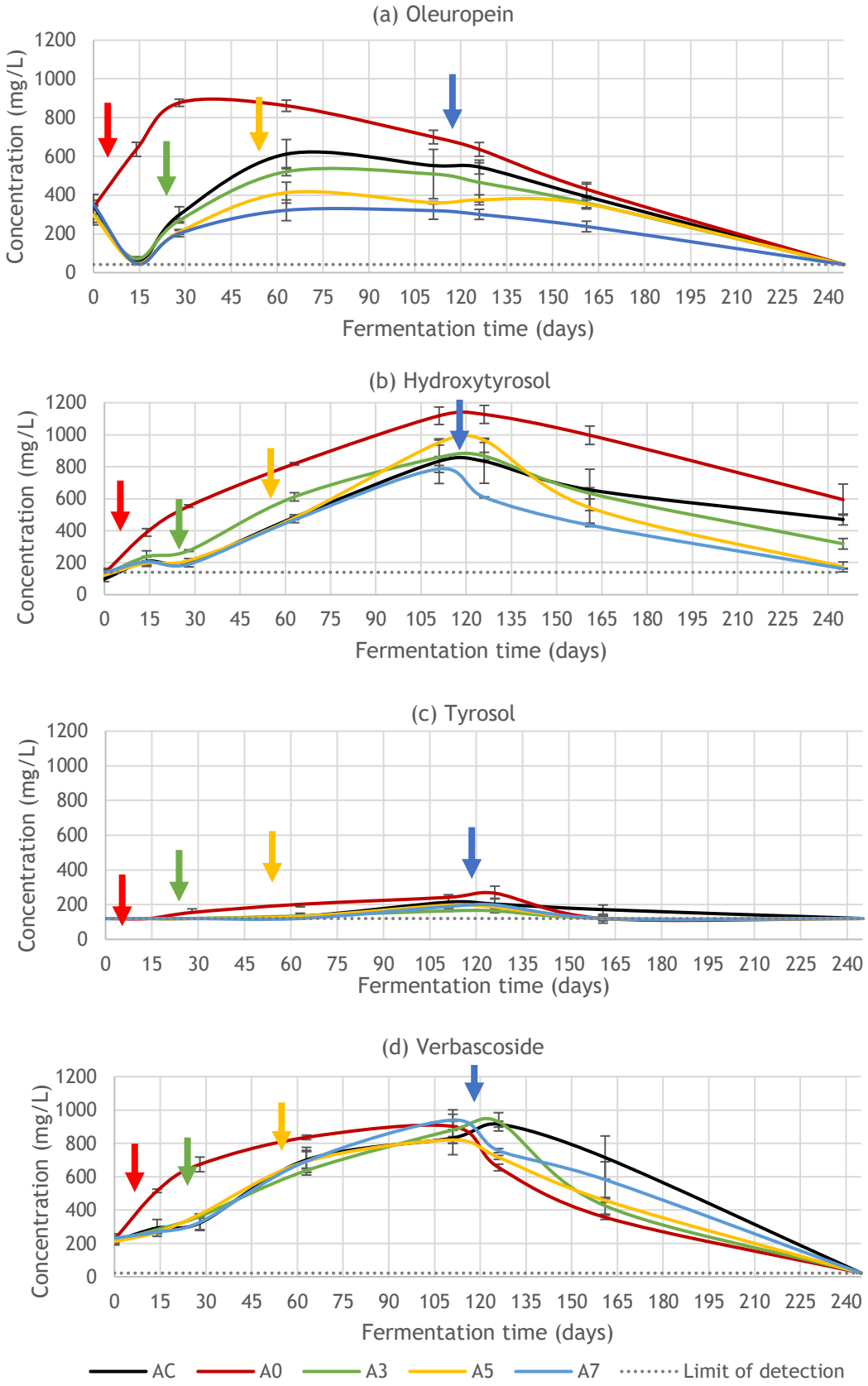

Figure 10. Phenolic profile of table olive brine throughout fermentation of plan A, (a) oleuropein, (b) hydroxytyrosol, (c) tyrosol, and (d) verbascoside. Arrows correspond to the moment of inoculation with *Lpb. pentosus* i106 to the experimental trial and the corresponding salt addition to all vessels.

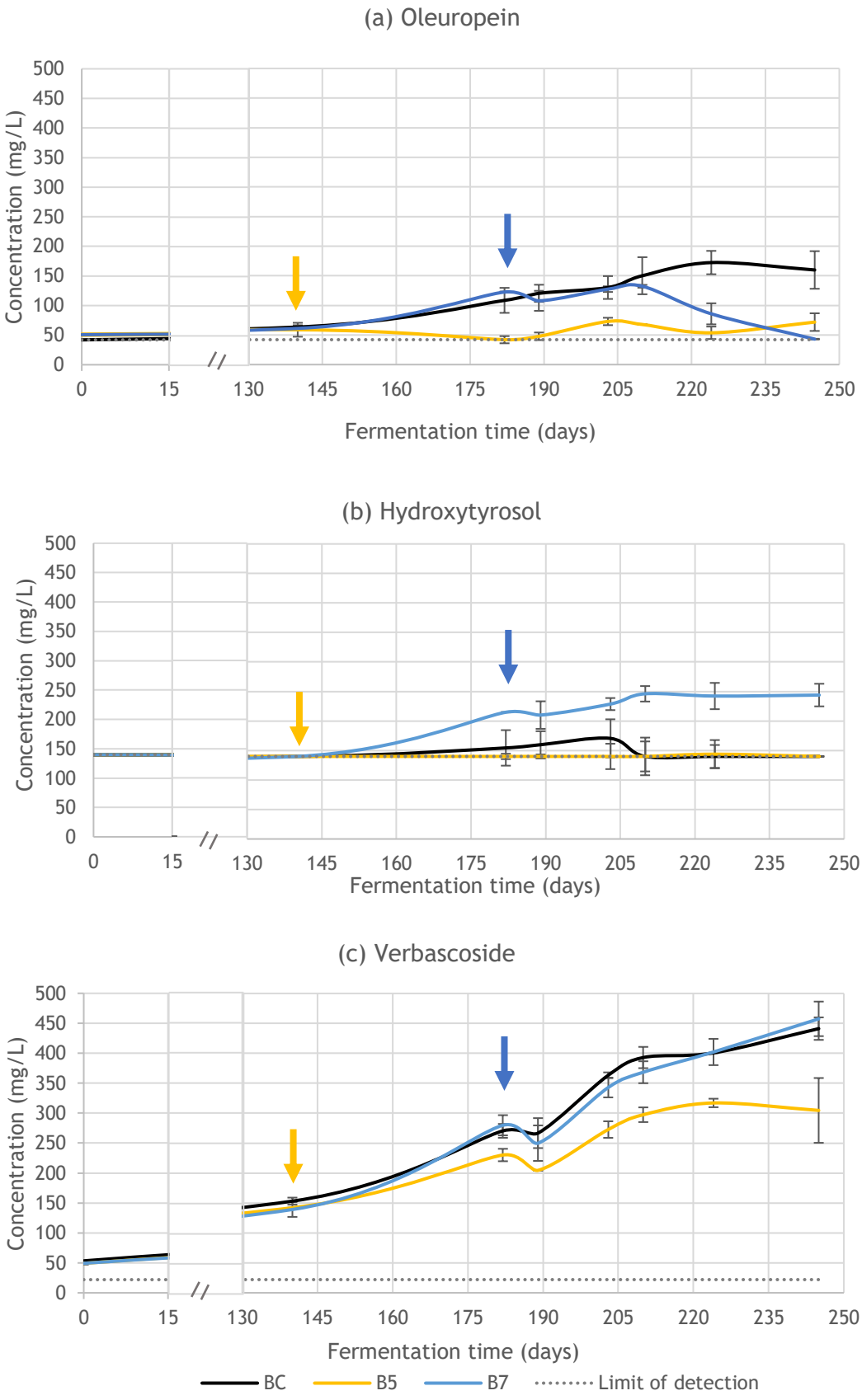

Figure 11. Phenolic profile of table olive brine throughout fermentation of plan B, (a) oleuropein, (b) hydroxytyrosol, and (c) verbascoside. Arrows correspond to the moment of inoculation with *Lpb. pentosus* i106 to the experimental trial and the corresponding salt addition to all vessels.

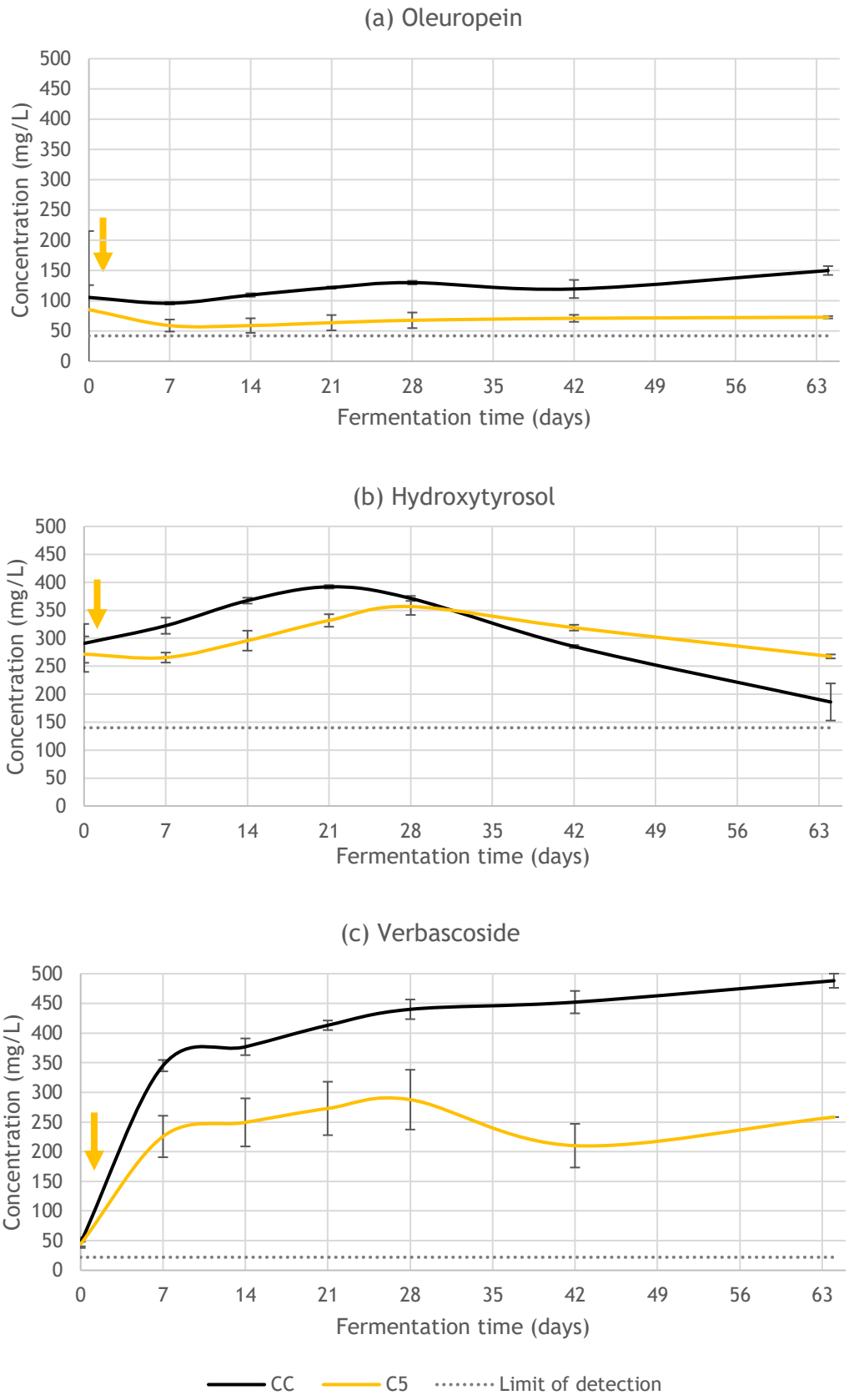

Figure 12. Phenolic profile of table olive brine throughout fermentation of plan C, (a) oleuropein, (b) hydroxytyrosol, and (c) verbascoside. Arrows correspond to the moment of inoculation with *Lpb. pentosus* i106 and the corresponding salt addition to all vessels.

3. Nutritional composition

Table 4 exhibits the table olives edible part nutritional profile from the three different plans followed by the end of the experiment - including moisture, ash, salt, proteins, fats, fibre, and carbohydrate content, and also the energetic value per each 100 g of edible part of table olives.

All nutritional parameters presented significant differences ($p<0.05$) between harvesting time (plan A and B from 2021; and plan C from 2020), and some between different protocols of addition of *Lpb. pentosus* i106. Moisture and protein contents were significantly higher in 2019/20 harvest (green colour) than 2020/21 harvest (turning colour); whereas fat was significantly lower in 2019/20 harvest than 2020/21 harvest. Regarding ash and salt content, the results did differ significantly between the three plans: plan C was the highest, followed by plan B and finally plan A. Ash content in plan A was higher when the salt percentage added was higher, but it did not differ among plan B or C. Concerning salt content, under plan A conditions, the control had lower results - and these were higher in trials A5 and A7; under plan B, no differences were recorded; while in plan C, the control was significantly lower. However, for plan A, some significant different differences were found: (i) fat content tended to decrease with time of addition of probiotic strain and salt concentration; and (ii) protein content exhibited an opposite tendency. Fibre content was similar in all trials, except for trials B5 and C5 - which exhibited the lowest and the highest values, respectively. Regarding carbohydrates, plan A was characterized by lower values, with the exception of the trial A7, and plan C the highest. Energetic values also differed between harvests, presenting significantly lower values for the 2019/20 harvest.

Table 4. Nutrional profile of table olives (content per 100 g of edible part of table olives), under the different plans.

	Trial	Content per 100 g of the edible part of the table olives							Energetic value (kcal/100 g)
		Moisture	Ashes	Salt	Proteins	Fats	Fibre	Carbohydrates	
Plan A	AC	67.97 ± 0.42^{abc}	3.22 ± 0.04^{a}	2.60 ± 0.15^{a}	0.92 ± 0.13^{ab}	22.21 ± 0.49^{c}	3.78 ± 0.11^{ab}	1.90 ± 0.06^{a}	218.73 ± 4.50^{bc}
	A0	66.28 ± 0.81^{a}	3.29 ± 0.05^{ab}	2.88 ± 0.01^{ab}	1.03 ± 0.05^{abc}	22.82 ± 0.06^{c}	4.46 ± 0.51^{ab}	2.13 ± 0.25^{a}	226.92 ± 2.36^{c}
	A5	67.90 ± 0.29^{abc}	3.46 ± 0.01^{bc}	2.95 ± 0.08^{b}	1.16 ± 0.15^{abc}	20.69 ± 0.48^{bc}	4.66 ± 0.44^{ab}	2.14 ± 0.21^{a}	208.68 ± 2.08^{b}
	A7	66.36 ± 0.35^{a}	3.49 ± 0.09^{c}	2.94 ± 0.02^{b}	0.89 ± 0.07^{a}	19.81 ± 1.30^{b}	5.84 ± 1.15^{ab}	3.20 ± 0.67^{abc}	207.70 ± 7.02^{b}
Plan B	BC	66.60 ± 1.22^{ab}	3.97 ± 0.03^{de}	3.70 ± 0.09^{c}	1.18 ± 0.17^{abc}	21.08 ± 0.32^{bc}	4.80 ± 0.50^{ab}	2.38 ± 0.25^{ab}	213.54 ± 1.56^{bc}
	B5	67.86 ± 0.23^{ab}	3.81 ± 0.02^{d}	3.64 ± 0.02^{c}	1.04 ± 0.12^{abc}	21.34 ± 0.02^{bc}	3.64 ± 0.25^{a}	2.32 ± 0.15^{ab}	212.72 ± 1.74^{bc}
	B7	67.12 ± 1.39^{ab}	3.86 ± 0.06^{d}	3.64 ± 0.08^{c}	1.13 ± 0.02^{abc}	20.73 ± 0.09^{bc}	4.43 ± 0.86^{ab}	2.73 ± 0.52^{abc}	210.91 ± 4.55^{b}
Plan C	CC	70.22 ± 0.02^{c}	4.07 ± 0.02^{e}	3.61 ± 0.06^{c}	1.34 ± 0.13^{c}	15.34 ± 0.44^{a}	5.98 ± 0.34^{b}	3.47 ± 0.19^{b}	167.89 ± 2.99^{a}
	C5	70.02 ± 0.82^{c}	4.12 ± 0.02^{e}	4.02 ± 0.06^{d}	1.47 ± 0.08^{c}	14.64 ± 0.38^{a}	9.41 ± 0.37^{c}	3.78 ± 0.15^{c}	171.58 ± 5.14^{a}
	p-value	0.003	< 0.001	< 0.001	0.009	< 0.001	< 0.001	0.002	< 0.001

Each value in the table represents the mean ± standard deviation of two independent assays.
$^{a-e}$ Means followed by different lowercase letters in each column differed significantly among trials ($p<0.05$) by Tuckey Post-Hoc comparison.

4. Sensory assessment

The sensory characteristics of all table olives prepared resorting to the different treatments were evaluated at the end of the process by 12 panellists according to IOC standards. **Figure 13** present the sensory analysis corresponding to plans A, B and C, encompassing table olive texture, flavour, saltiness, bitterness, acidity and abnormal flavours.

4.1. Plan A - Olive fermentation without sweetening

The five trials did not unfold sensory differences for salty (ca. 4.5) and abnormal flavours (ca. 4.5 points). Differences of texture between trials were found; it was better for trials when strain is added earlier (ca. 4), and then decreased to 3 or 2 for A5 and A7, respectively. Flavour quality was worse for the trial A7 (4) when compared to the other trials that were essentially equivalent (6). Bitterness increases as the strain is added earlier – 6 for AC and A0, 5 for A3, 4 for A5, and 3 for A7. A similar pattern was found for the acidity of table olives, 5 for AC and A0, 4 for A3, and 3 for A5 and A7.

4.2. Plan B - Olive fermentation after previous sweetening

In addition to salty (ca. 5) and abnormal flavours (ca. 4.5 points), the texture did not exhibit significant differences (ca. 5). The quality of flavour was better, and equal to BC and B5 (ca. 6.0) when compared to the B7 (4.5). Bitterness decreased with addition of *Lpb. pentosus* i106, either in B5 or B7 (ca. 3) - with a value of 5 for the control. Acidity also decreased with addition of the strain i106 (ca. 3.5) when compared to the control (ca. 5.5).

4.3. Plan C – Fermentation with ready-to-eat olives

No significant differences between the two trials could be found for the 6 sensory parameters.

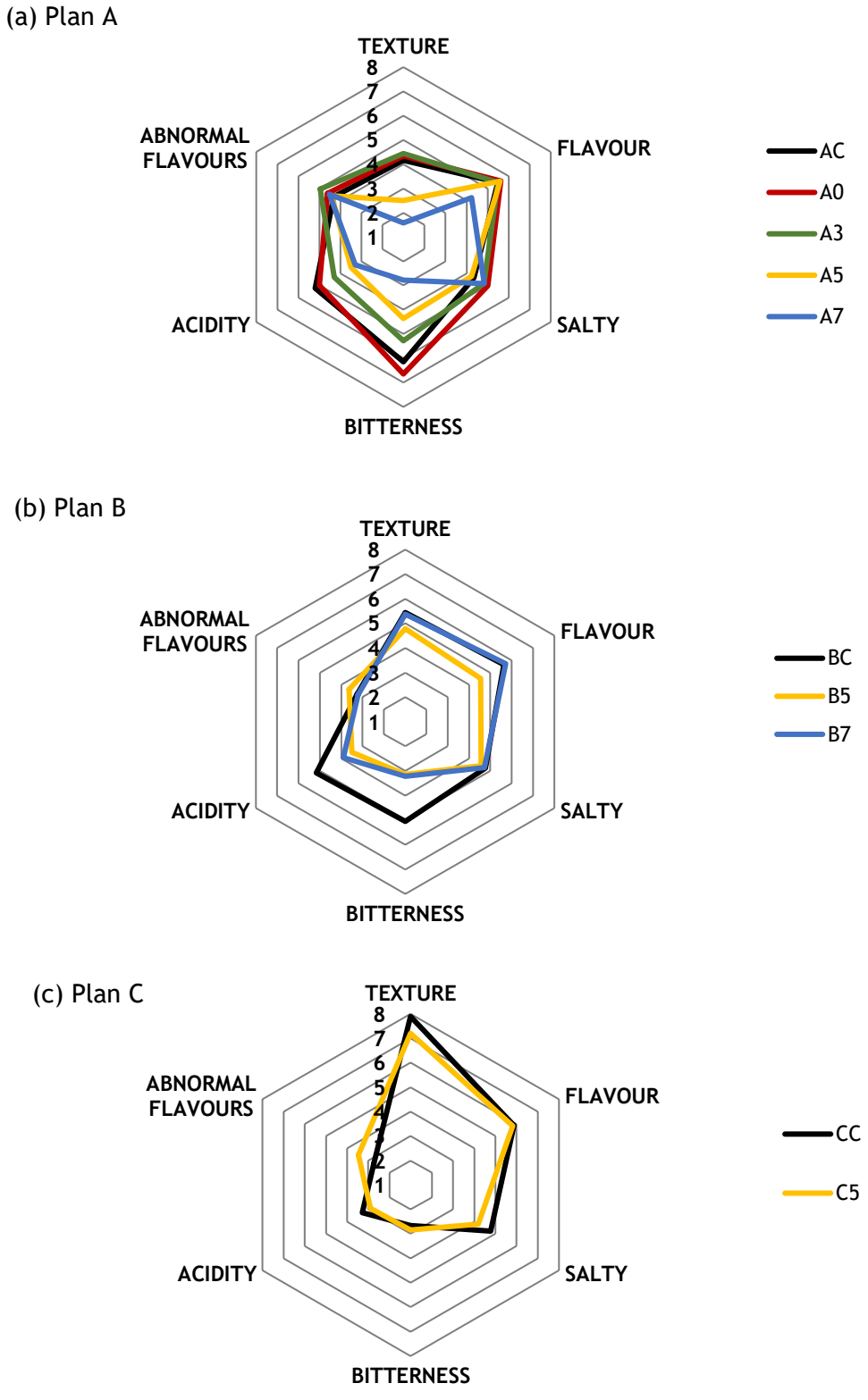

Figure 13. Sensory analysis of *Cobrançosa* table olives fermented under (a) plan A, (b) plan B, and (c) plan C.

4.4. Overall sensory quality

Figure 14 allows comparison of the overall eating quality (OEQ) between plans. One found that points given to OEQ under plan C (green olives) were statistically higher than those under plans A and B (both with turning colour olives). From **Figure 14**, one also verified better evaluations for plan B (i.e., when the addition of *Lpb. pentosus* i106 is made after sweetening) than for plan A. For plan B, the highest value for OEQ was obtained for B7. However, for plan A, the opposite was observed, i.e. a lower OEQ was observed for the last trial (A7).

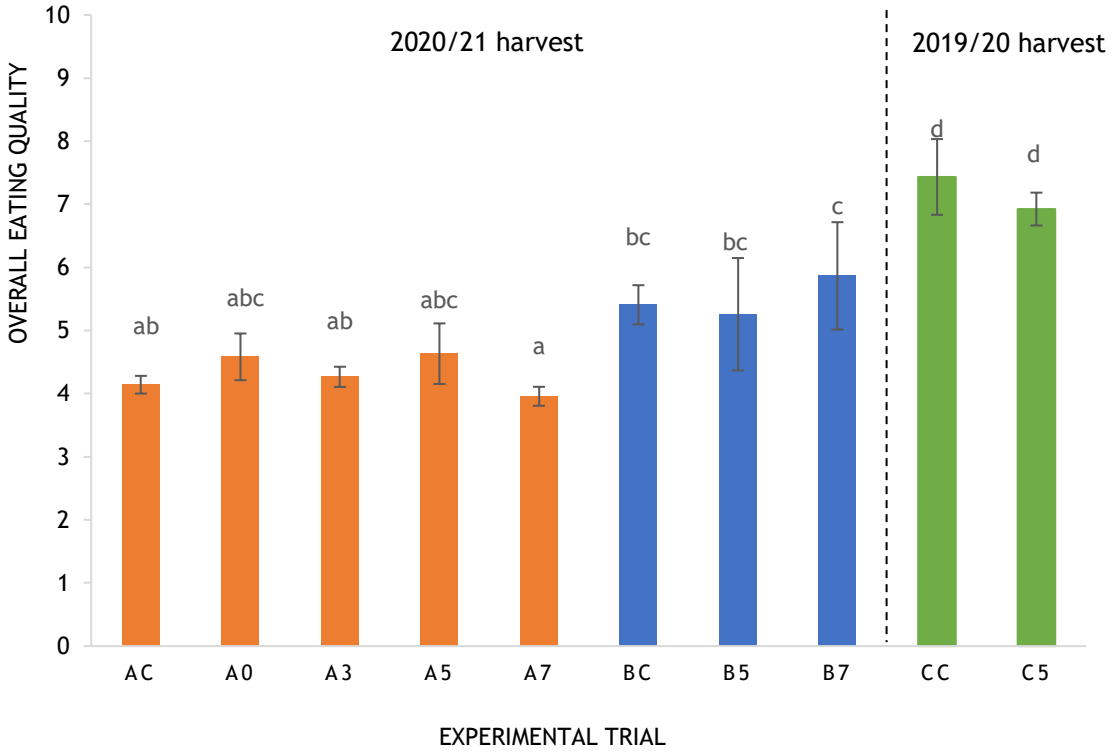

Figure 14. Overall eating quality of *Cobrançosa* table olives fermented under the different plans. Each column represents the mean ± standard deviation of two independent trials. [a-d] Means followed by different lowercase letters in each column differed significantly among trials ($p<0.05$) by Tuckey Post-Hoc comparison.

IV. DISCUSSION

The maturation degree of table olives has a significant impact in nutritional composition; therefore, plan C should be discussed separately from plans A and B. In order to discuss all results obtained as a whole - and focusing on the main objective of this study, i.e. the production of *Cobrançosa* table olives with a probiotic character via addition of an endogenous LAB culture, one should look for the plans that produced a better sensory evaluation, and (if possible) for the best results attained within each plan. This way, it will be easier to justify the profiles of the other parameters analyzed (such as microbial and physicochemical data, namely with regard to oleuropein degradation).

Nutritional properties of table olives - besides varying, in general, according to cultivar or applied processing method, some agriculture practices, preharvest factors such as irrigation and fruit ripening stage, agroclimatic conditions and geographical origin may also play a role (Rocha *et al.*, 2020; Rodrigues *et al.*, 2022). In this study, it was possible to control the cultivar and fruit ripening stage (which are related to the harvest decision). Regarding the cultivar, previous studies on cv. *Cobrançosa* exhibited nutritional values very similar to those in this study – of fat content (16.5-23%), protein content (1.2-1.4%), carbohydrate (2.1-3.5%), fibre (1.2-2.8%) and ash (4.2-4.4%), corresponding to energetic values of 180-220 kcal/100 g (Malheiro *et al.*, 2012; Pires-Cabral *et al.*, 2018; Prata, 2015). However, the colour of the olive - which is directly related to the maturation degree of the drupe, has some influence upon the nutritional value of the final product. As explained by Rocha *et al.* (2020), as the maturation of olives gradually occurs, the fat content of the olive flesh, and consequently the energetic value increase simultaneously along with a decrease of water content; this corroborates the results of this study. An amount of 50 g of table olives can provide about 5% and 15 to 30% of the daily energy and total lipid requirements, respectively, considering the 33 – 67 g of lipids/day recommended amount (Pires-Cabral *et al.*, 2018). Fibre content also differed between maturation degree, being higher in the 2019/20 harvest olives (green olives); this happens because during the maturation process the activity on the cell wall leads to fibre degradation, namely cellulose and hemicellulose (Galanakis, 2011). Higher fibre content might give these olives an additional nutritional value, since they are important for gastrointestinal regulation, dietetic treatments, or even promotion of the reduction of incidence of some diseases – as long as around 5 g/100 g of table olives (Prata, 2015). The ash content is related to the minerals present in the table olives and salt concentrations of the brine that

diffuse into the table olive flesh during fermentation; hence, lower ash content occurs at higher maturation, and is related to lower salt levels (Malheiro *et al.*, 2012; Prata, 2015) - in agreement with the nutritional values obtained in the 2020/21 harvest (turning colour olives), which presented lower ash and salt content.

Based on overall quality sensory evaluation, it is possible to infer that the best plan was plan C. The first reason for this is probably the shorter maturation time of table olives; the same preference of green olives over turning colour olives was also reported by Hurtado *et al.* (2009) on *Arbequina* natural table olives. The texture did also differ significantly among plans, as shown in the Results section; these differences can be explained by hydrolysis of cell wall pectic polysaccharides during the fermentation process, which results in loss of structural coherence of olive tissues leading to softer textures. When salt is added too soon, it is more likely to soften the olives, as per the induced osmotic dehydration process that gradually damages the cell tissues (Campus *et al.*, 2017); too soft table olives are often associated with a negative appreciation – as mentioned by Prata (2015). Second, it is possible to infer that the sensory quality of the final product is not affected when *Lpb. pentosus* i106 is added to green table olives as additive at packaging; this is understandable by the final similarities between trials, in terms of physicochemical and debittering process (oleuropein depletion) – considering that the LAB strain grows significantly during the first week, and remains thereafter, but also starts to grow after 40 days in the control trial.

In view of the above, the discussion will hereafter focus on the differences between plans A and B. The existence of a sweetening stage had a positive impact on the overall quality of final turning colour table olives; therefore, this stage should be maintained within the fermentation process, for having no interference with the addition of a LAB probiotic culture.

For plan B, the control and the two protocols exhibited the same level of texture and salt - as expected, due to the similar maturation degree of table olives and equal time of salt addition; as well as abnormal flavours - which is a good result. The control appears to be the preferred trial, mainly because of higher acidity and bitterness; these two sensory parameters can thus be claimed as possessing a higher relevance toward the evaluation of sensory properties of this particular cultivar. The sensory analysis agrees with the results obtained for pH, TTA, and phenolic compounds – trials with higher acidity and bitterness presented lower pH, higher TTA, and higher phenolic content, namely oleuropein. Although the addition of *Lpb. pentosus* i106 significantly

increase the numbers of LAB in both brine and olives, and consequently the pH and biochemical profiles, the spontaneous growth of LAB compensates once again these variations at a later stage of the process in the control - in such way that the final pH and biochemical concentrations of the control contribute to a better overall sensory quality, mainly due to the pH being lower, and TTA and oleuropein higher. These two facts can be rationalized by the fact that, in trials B5 and B7, *Lpb. pentosus* i106 helps accelerate the process of: (i) production of lactic acid, which is then converted to other organic acids with the help of yeast; and (ii) oleuropein degradation (Anagnostopoulos and Tsaltas, 2022). Finally, the evaluation of flavour was similar between control and trial B5 - yet more information on flavour compounds in table olives should be collected in the future, besides the phenolic compounds that confer the typical bitterness to the olives. During fermentation and storage, yeasts can produce compounds such as ethanol, glycerol, higher alcohols, esters, and other volatiles playing an important role in flavour and aroma generation, but also in texture maintenance (Arroyo-López *et al.*, 2008; Mujdeci and Ozbas, 2021). Regarding LAB, their metabolism releases compounds, such as acetoin and diacetyl, that provide buttery flavours; while the organic acids generated (e.g. lactic acid and acetic acid) are related to sour flavours (Sedó Molina *et al.*, 2022).

If the case where sweetening stage is not performed (plan A), no significant differences are obtained for overall eating quality; hence, if probiotic table olives first thinking relies on bitterness and acidity, the best moment to add the strain is at the beginning (trial A0) - based on similarity of all sensory parameters to the control – and in this case one can speak of a probiotic starter culture. Considering that control and trial A0 got the same evaluations for flavour, texture, and abnormal flavour, when the addition of the strain occurs later in the fermentation process, the sensory assessment becomes worse.

After the possible relations between overall and individual parameters of sensory properties of *Cobrançosa* table olives have been discussed. including the effect of different protocols - with microbiological profiles different from the control, as expected, leading to distinct pH and phenolic compound profiles, these subjects shall be discussed to further detail.

Microbial evolution revealed that microorganism populations, either LAB or yeasts, are in higher significance in brine than in table olives, differing by 1 up to 2 log, irrespective of the year of harvest. The studies by Anagnostopoulos *et al.* (2020a) on green cracked Cypriot table olives, Pereira *et al.* (2008) on Portuguese natural olives

cv. Galega, and Kumral *et al.* (2009) on Turkish natural black table olives *cv. Gemlik* also found this difference. Said finding was apparently related to the high concentration of phenolic compounds in the pulp of olives, thus, associated to high antimicrobial activity that triggers to microbial inhibition (Anagnostopoulos *et al.*, 2020a); as well as LAB and yeasts that tend to be typically found in the pores, lesions, lenticels, or irregularities of olive surface, which might led them to the brine instead of the olive cells (Anagnostopoulos and Tsaltas, 2022; De Bellis *et al.*, 2021).

Furthermore, the dominant microflora in control trials throughout the process were yeasts; LAB were only detected in advanced stages of salting. This was expected, as a consequence of the study Reis *et al.* (2022), previously performed within this research group laboratory. However, this study concluded that yeast populations keep their growth throughout the process irrespective of the increase of salt content, or addition of high counts of *Lpb. pentosus* i106 (inoculum up to 8 log CFU/mL in brine, as mentioned in section II-1.2); on the other hand, LAB take longer to adapt to the new environmental conditions as the percentage of salt increases, as found in plans A and B (Rocha *et al.*, 2022). Nevertheless, no matter the plan, probiotic LAB strain i106 at the end of the process is present to significant numbers in brine (ca. 7-8 log CFU/mL) and olive (ca. 6-7 log CFU/g), as intended. Plan B helps understand that, if the purpose is having probiotic table olives throughout fermentation, the optimum salt content must be 5-6%. This agrees with Penland *et al.* (2022), who showed that LAB populations were below the detection limits in all fermentations due to the high salt content in brines (8-10%). Tassou *et al.* (2002) also showed that 4 and 6% NaCl brines favoured LAB growth, when compared to 8% NaCl brines. Plan C emphasizes that the probiotic LAB i106 was alive after 1 month of storage, which can guarantee table olives probiotic potential during that period; nevertheless, this is a short time for typical storage, namely for producers and wholesalers, so further analysis would be necessary throughout time to infer the strain viability and stability.

Spoilage or even pathogenic species could develop during the first stages of fermentation, but usually decay rapidly to yeasts and LAB - since they are more sensitive to salt concentration and acidification of brines, brought about by the metabolic activity of LAB (Campus *et al.*, 2018). This statement agrees with this study. In Plan A – before any sweetening or salting, either in brine or in table olives, those populations were significantly higher at the beginning of the experiment, lowering after addition of salt and strain *Lpb. pentosus* i106 in each trial, and reaching values below the detection limit as long as the pH lowers and the salt and titratable acidity increase. In this case, it seems that the addition of probiotic LAB i106 helped to reduce

Enterobacteriaceae faster than control (at lower salt contents), due to its own co-aggregation with pathogens such *Escherichia coli* ATC25922 - as reported in the study by Coimbra-Gomes *et al*. (2022b). Looking at the salt content and pH values at the initial period, plans B and C, and the corresponding values of plan A at same time, *Enterobacteriaceae* were not expected, as effectively observed in this study. Servili *et al*. (2006) also observed this decrease of *Enterobacteriaceae* populations, as *Lpb. pentosus* populations increased in black table olives (*cv. Itrana* and *Leccino*). Regarding the three groups of microorganisms, Anagnostopoulos *et al*. (2020b) presented very similar microbial evolutions along fermentation of natural whole and cracked *Picual* table olives, inoculated with a *Lpb. plantarum* strain - even similar differences between inoculated and control trials; and concluded that inoculation can, in fact, accelerate and favour the elimination of unwanted microorganisms.

With these experiments, it was found that yeasts are not affected at all during *Cobrançosa* fermentation, but the stage of the process when salt is added, as well as its concentration, are determining factors upon growth rate and biomass reached by the probiotic LAB strain i106. How this LAB growth was reflected upon the physicochemical properties and degradation of oleuropein when compared to the control will be discussed next.

As expected, salt content in brines increased in a discrete way throughout time; and was equal within trials of each plan. However, at later stages of fermentation, it was possible to observe that brine salt content decreased and could not exceed a certain level – becoming constant, despite the addition of more salt; this was also reported by Lanza *et al*. (2020) for *Olea europaea L. Itrana cv. o*lives, and can be explained by the osmotic equilibrium reached between brine and table olive cells.

The values of pH profiles were consistent with *Lpb. pentosus* i106 growth. In each time of sampling, trials that have been inoculated earlier with probiotic LAB culture did always exhibit lower pH and higher titratable acidity. Independently of the plans, the profiles of pH for trials A0 and A3 were similar - as expected due to the tolerance to pH and similar growth rate of these strains below 5% of salt, as claimed by Rocha *et al*. (2022); Penland *et al*. (2022) exhibited a similar pH tendency on Nyons black table olives, beginning at 5.4, followed by a decrease throughout fermentation until 4.4, remaining constant afterwards; on the other hand, the profiles of pH for the trials A5 and A7 were the opposite - lower tolerance to higher salt contents led to poorer adaptation of the strain to the environment, thus causing lower growth rate that resulted in higher pH values and lower acidification; this was also found by

Anagnostopoulos *et al.* (2020a), where LAB growth seems to have been hampered by salt-tolerant yeast species, thus resulting in a less acidic product.

Titratable acidity values have increased throughout the first days of fermentation, from 0.15-0.56, 0.05-0.21, and 0.14-0.29 g/100 g lactic acid, in plans A, B and C, respectively, namely for those trials where inoculation was performed earlier. This is mainly due to: (i) LAB activity on the production of organic acids, such as lactic acid; (ii) diffusion and solubilization of those compounds from the table olive tissues; and (iii) increase of free fatty acids present in the olives, or produced by microorganisms (Mateus *et al.*, 2016). Similar results were reported by Lanza *et al.* (2020) - 0.17 – 0.30 g/100 g lactic acid; and by Mateus *et al.* (2016) – 0.1-0.6 g/100 g lactic acid. Nevertheless, by the end of the experiment, titratable acidity values suffered a significant decrease in all trials. Acidity decrease (pH increase) is justified by yeast dominance over LAB populations that also metabolize organic acids - as reported by Anagnostopoulos *et al.* (2020b), Aponte *et al.* (2010), Panagou *et al.* (2003), Papadelli *et al.* (2015), and Reis *et al.* (2022); in fact, in plans B and C, where titratable acidity is lower, yeasts populations exist to quite similar values, compared to LAB populations. As stated in Anagnostopoulos *et al.* (2020b), although the *Lpb. pentosus* reveals to have some salt tolerance, the capacity of the strain to maintain a homofermentative metabolism (hence, producing e.g. acetic acid) under stress conditions is reported to be lower when compared to strains such as *Lpb. plantarum*; these species are facultative heterofermentative and shift from homo- to heterofermentative metabolism depending on environmental stress conditions such as oxygen and nutrient limitation, salt concentration, and low pH levels.

As mentioned before, green table olives received better sensory scores than the turning colour; it was also mentioned that bitterness is an intrinsic requirement for good quality *Cobrançosa* table olives. This is in agreement with the fact that, during the growth phase of olives, the total phenol concentration, in particular oleuropein, increases to a maximum level at the green maturation phase, followed by a significant reduction; however, there is continuous synthesis of these compounds until maturity (Rocha *et al.*, 2020). In agreement with this study, Sousa *et al.* (2014) also identified oleuropein as the main phenolic compound in the first stages of maturation, decreasing significantly during ripening - while hydroxytyrosol became predominant at intermediate and long maturation stages. Although oleuropein is the major component responsible for the bitter taste in olive fruits, it is known that it tends to disappear throughout fermentation, through bacterial activity, or endogenous and chemical hydrolysis (see **Figure A6** in **Appendix IV)**, e.g. by β-glucosidase enzymatic activity,

leading to less bitter phenolic compounds, such as hydroxytyrosol; this turns olives edible, and increases acceptance by the consumer (Penland *et al.*, 2022; Rocha *et al.*, 2020). This point was confirmed during some periods of fermentation, but was more evident in the trials where LAB probiotic culture i106 was inoculated first.

Considering all phenolic compounds analysed, and looking at the plans, the concentration evolution throughout fermentation follows previous trends studied by this research group (Reis *et al.*, 2022). Furthermore, the LAB strain i106 has been previously proven to have the ability to degrade oleuropein (Rocha *et al.*, 2022). Oscillations in phenolic compounds of plan A are also highly affected by the washing and sweetening processes; as observed in **Figure 10(a)**, after two weeks, a significant decrease in oleuropein content is observed in all trials, except A0. This is justified by the lack of washing of the said trial prior to the addition of the probiotic strain. Although the goal was to reproduce an actual starter culture, this procedure led to higher phenolic compound concentrations throughout all the experiment for this trial, as outlined in **Figure 10**. The importance of the sweetening process is once again highlighted by the much lower overall phenolic concentrations found in plans B and C, when compared to plan A - since both have been submitted to sweetening, while plan A did not; by washing the olives, phenolics content that have diffused away from the flesh to the brine are eliminated (Reis *et al.*, 2022).

Additionally, it was expected that inoculated vessels would present higher oleuropein degradation into other phenolic compounds (hydroxytyrosol, verbascoside, or tyrosol) than those not inoculated (control trials); this was more noticeable in plans B and C, where *Lpb. pentosus* i106 strain was added. A similar LAB debittering activity during fermentations was reported by Lanza *et al.* (2020).

Verbascoside in this study was found to increase alongside with hydroxytyrosol decrease at later stages of fermentation - being more noticeable in plans A and C. The formation of verbascoside is apparently related to partial degradation of oleuropein, thus explaining the later appearance of this compound in olive fruits; but is also linked to the conjugation of hydroxytyrosol with organic acids during ripening (Rodrigues *et al.*, 2022). Therefore, verbascoside increase should happen alongside with oleuropein and hydroxytyrosol decrease (Termentzi *et al.*, 2015). The verbascoside increase with time was also reported by Sousa *et al.* (2014) for mature fruits of *cv. Cobrançosa*; while in immature fruits, this compound was not detected. Conversely, this study found this compound in green olives (plan C) to similar levels in turning colour olives (plan B).

However those results were obtained for table olive dry matter, while this study determined the phenolic compounds present in table olive brines.

Tyrosol, besides being reported in plan A during the fermentation, was always present to very low concentrations; in plans B and C, its concentration was constantly below the detection limit. This compound was also found by (Chytiri *et al.*, 2020) in natural black olives (*cv. Kalamata* and *Conservolea*), and by Kaltsa *et al.* (2015) in their study on green (*cv. Chalkidikis*) and black (*cv. Kalamata*) table olives pulp. The process of oleuropein and ligstroside (another phenolic compound, commonly present in table olives) released by lysis takes place in two phases (see **Figure A5**; **Appendix 1**): first, the hydrolysis of the glycosidic bond by ß-glucosidase activity, with formation of oleuropein-aglycons or ligstroside-aglycons; second, the hydrolysis of the aglycons by esterase, with formation of elenolic acid and hydroxytyrosol (from oleuropein-aglycon) or tyrosol (from ligstroside-aglycon). The low values in plan A and the lack of tyrosol in plans B and C, agree with the findings by Coimbra-Gomes *et al.* (2022b) – all native LAB strains from *Cobrançosa* (*Lpb. pentosus* and *Lpb. paraplantarum*) revealed low esterase activity and high ß-glucosidase activity, the former being the main responsible for tyrosol production (Lanza *et al.*, 2020; Termentzi *et al.*, 2015). In addition, the presence of tyrosol in plan A but not in B or C is, once again, justified by the lack of washing and sweetening, which leads to higher phenolic compound concentrations.

It is important to notice that final values for phenolic compounds below the detection limit are justified by the intrinsic complexity of the sample matrices by the end of the experiment - which hindered correct visualization of peaks in the chromatograms (see **Figure A7** in **Appendix IV**) - and consequently hampered the quantification of compound concentration.

V. CONCLUSIONS

The maturation degree of *Cobrançosa* table olives has an important impact on the overall eating quality of this type of cultivar – especially on texture; hence, they should be treated in the final stage of green before turning colour. It is suggested to define an index of maturation for future process standardization. One also suggests reduction of salt content to 5% in the final product, to enhance the health benefits. One also concluded that, it is possible to obtain a final product overall eating quality similar to the control (obtained by natural fermentation), with probiotic characteristics, by 1 up 2 months after adding *Lpb. pentosus* i106 at packaging stage. It was proven that a probiotic starter culture comprising this strain i106 has higher probability of not functioning *in loco* for this type of process, as long as sweetening has to be applied.

This study results indicated that, upon inoculation of *Lpb. pentosus* i106, both LAB and yeast microorganisms were in similar concentrations – besides unfolding populations at constant levels throughout the experiment; however, LAB strain growth is hindered by high salt percentage addition (above 5%). The elimination of *Enterobacteriaceae* is rapidly obtained as salt content increases, pH decreases, and total acidity increases.

Bitterness is a sensory parameter with a great impact upon the overall quality of *Cobrançosa* table olives. Even though the phenolic compound degradation, namely oleuropein, did occur gradually as expected, it was achieved faster in inoculated trial - thus producing higher levels of hydroxytyrosol and verbascoside, and tyrosol – meaning less bitterness and poorer sensory preference.

Nutritionally, major differences were found; while in the 2020/21 harvest, turning colour olives exhibited higher fat content and lower water content, the 2019/20 harvest (green) presented the opposite, along with higher fibre content. In any case, both revealed to be a great source of healthy compounds, revealing no major differences between trials of each plan.

VI. FUTURE WORK

In terms of studies on microorganisms, further validation of probiotic strains shall be performed via *in vivo* trials, with more complex animal or human systems aimed at gaining a deeper understanding of their potential health-promoting features for humans. Considering that yeasts were dominant microorganisms over all the fermentation process, future studies should also rely on isolation and characterization of yeast strains from *Cobrançosa* cultivar toward the development of conditions also for multiplication of yeast probiotic starter strains; or even hybrid starters, by combining LAB and yeasts, to mimic the natural microbiota of olives.

Further technological studies could also look for new fermentation strategies, meant to favour survival and dominance of other LAB probiotic starter strains (already characterized by this research group). For example, the use of microencapsulated microorganisms, the use of biofilms, or the use of post probiotics may be tested – considering that one major conclusions of this study points at adding the culture during packaging. One also suggests that temperature monitorization of table olive brines along fermentation should be done; besides salt, temperature is one of the parameters with higher influence upon fermentation, with major impact upon microbiological growth, and solute diffusion rates between table olives and brine.

To better understand the high value to health, deeper studies on nutritional composition should be done – encompassing polyunsaturated and monounsaturated fatty acids and amino acids for adults (threonine, valine, leucine, isoleucine, phenylalanine, and lysine), and for children (arginine, histidine, and tyrosine).

Finally, and in terms of environment awareness, new approaches for the treatment of wastewater produced by table olive industries might also be developed - in order to have sustainable healthy eco-friendly products, e.g. compounds derived from phenolic, hydrocarbon, and sugar fractions, meant for recovering and reuse, thus upgrading the wastewater.

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APPENDIX

I. Table olives samples

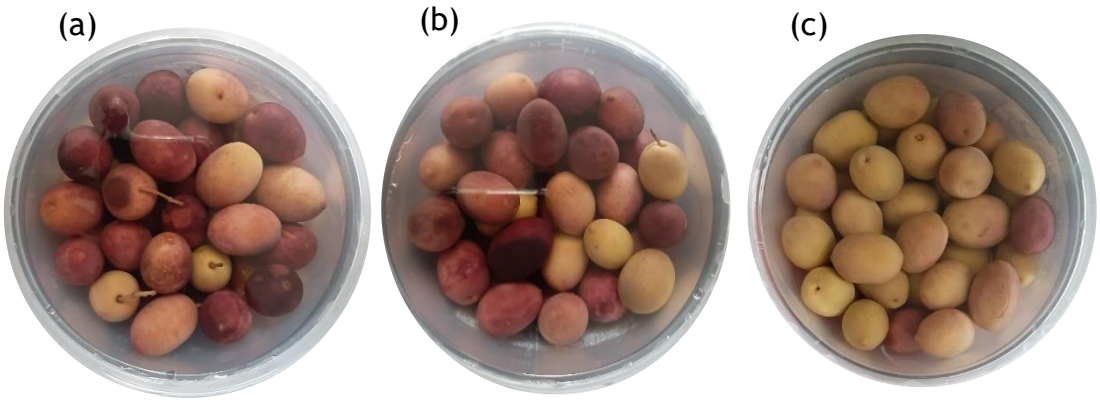

Figure A1. *Cobrançosa* table olives samples from (a) plan A, (b) plan B, and (c) plan C, by the end of the experiments selected for sensory analysis.

II. Microbiological analysis

Table A1. Complete microbial (LAB) monitoring results for brine and *Cobrançosa* olives fermented under plan A.

Fermentation time (days)	Trial									
	AC		A0		A3		A5		A7	
	Mean	STD	Mean	STD	Mean	STD	Mean	STD	Mean	STD
Brine (log CFU/mL)										
0	n.d.		n.d.		n.d.		n.d.		n.d.	
7	3.89	0.00	5.52	0.44	5.53	0.00	5.84	0.00	5.00	0.00
14	6.20	0.00	6.52	0.02	6.58	0.00	6.68	0.00	6.79	0.00
21	5.99	0.00	8.06	0.07	6.27	0.00	5.96	0.00	5.84	0.00
28	n.d.		7.17	0.66	6.50	0.29	n.d.		n.d.	
35	n.d.		5.21	0.64	4.87	0.22	n.d.		n.d.	
56	n.d.		7.20	0.55	8.03	0.00	n.d.		n.d.	
63	n.d.		6.97	0.22	7.99	0.00	n.d.		n.d.	
77	n.d.		7.12	0.15	6.97	0.07	4.24	0.02	n.d.	
98	n.d.		6.88	0.22	6.11	0.28	4.72	0.60	n.d.	
119	n.d.		6.28	0.17	6.91	0.06	5.49	0.44	n.d.	
126	n.d.		5.10	0.02	6.03	0.06	7.41	0.61	n.d.	
161	n.d.		6.61	0.48	6.50	0.17	6.61	0.14	6.54	0.00
245	2.96	0.00	7.53	0.22	8.04	0.86	7.49	0.23	7.54	0.05
Table olives (log CFU/g)										
0	n.d.		n.d.		n.d.		n.d.		n.d.	
7	n.d.		2.71	0.82	2.25	0.72	1.40	0.44	1.40	0.78
14	3.76	0.43	6.20	0.18	4.12	0.80	4.40	0.07	3.86	0.09
21	4.13	0.49	5.68	0.06	4.41	0.34	4.92	0.11	4.01	0.09
28	3.84	0.17	5.00	0.43	4.93	0.10	4.55	0.20	3.67	0.31
35	2.50	0.71	4.81	0.45	5.46	0.06	3.09	0.29	2.48	0.60
56	2.15	0.00	4.84	0.01	5.36	0.20	n.d.		2.86	0.00
63	n.d.		4.81	0.30	5.65	0.04	n.d.		n.d.	
77	n.d.		4.36	0.20	4.73	0.44	3.90	0.94	n.d.	
98	n.d.		5.31	0.44	4.19	0.44	4.31	0.93	n.d.	
119	n.d.		4.78	0.00	4.67	0.26	4.75	0.04	n.d.	
126	n.d.		4.17	0.18	5.01	0.29	6.28	0.33	n.d.	
161	n.d.		5.17	0.64	4.27	0.31	5.13	0.25	6.03	0.10
245	n.d.		5.95	0.18	5.81	0.17	6.14	0.20	6.79	0.07

STD – standard deviation;
n.d. – not detected (below the detection limit; < 1 log CFU/mL for brine and < 1.4 log CFU/g for table olives).

Table A2. Complete microbial (yeasts) monitoring results for brine and *Cobrançosa* olives fermented under plan A.

Fermentation time (days)	Trial									
	AC		A0		A3		A5		A7	
	Mean	STD	Mean	STD	Mean	STD	Mean	STD	Mean	STD
Brine (log CFU/mL)										
0	n.d.		n.d.		n.d.		n.d.		n.d.	
7	5.76	0.00	5.91	0.05	5.82	0.00	5.99	0.00	5.00	0.00
14	5.72	0.00	6.84	0.12	5.89	0.00	5.78	0.00	6.00	0.00
21	6.12	0.00	6.51	0.20	5.96	0.00	5.96	0.00	5.93	0.00
28	6.22	0.00	6.14	0.02	6.65	0.13	6.24	0.00	6.18	0.00
35	6.23	0.00	6.04	0.00	6.14	0.05	6.11	0.00	5.97	0.00
56	6.18	0.00	6.15	0.23	6.27	0.03	5.89	0.00	5.88	0.00
63	6.18	0.00	6.19	0.19	6.19	0.18	6.12	0.39	5.63	0.00
77	6.30	0.00	6.06	0.01	5.69	0.03	5.77	0.07	6.20	0.00
98	6.34	0.00	5.83	0.08	5.69	0.16	5.16	0.03	6.25	0.00
119	6.34	0.00	6.11	0.29	6.22	0.12	5.88	0.05	6.85	0.00
126	6.30	0.17	6.04	0.07	5.90	0.17	5.87	0.16	7.07	0.01
161	6.13	0.04	5.84	0.15	6.06	0.13	5.67	0.09	5.34	0.08
245	6.11	0.06	5.72	0.32	6.01	0.00	5.94	0.00	5.57	0.07
Table olives (log CFU/g)										
0	n.d.		n.d.		n.d.		n.d.		n.d.	
7	3.84	0.33	3.73	0.26	3.72	0.76	4.01	0.27	3.67	0.09
14	4.09	0.27	5.56	0.17	4.26	0.08	4.37	0.04	4.25	0.17
21	4.44	0.13	4.20	0.10	4.16	0.01	4.35	0.01	4.36	0.14
28	4.20	0.07	4.09	0.23	4.45	0.07	4.11	0.09	4.15	0.18
35	4.30	0.05	3.92	0.04	4.51	0.01	4.35	0.19	4.45	0.01
56	3.54	0.10	3.19	0.24	3.14	0.93	4.02	0.21	4.59	0.23
63	4.27	0.16	4.15	0.07	3.98	0.09	4.46	0.06	4.07	0.55
77	4.31	0.12	4.33	0.05	3.99	0.24	4.74	0.03	4.29	0.02
98	4.09	0.04	4.10	0.36	4.63	0.12	3.78	0.62	4.80	0.39
119	3.40	0.40	3.93	0.50	4.07	0.01	3.95	0.09	4.59	0.17
126	4.16	0.08	3.79	0.37	3.85	0.32	4.13	0.12	4.61	0.02
161	4.25	0.12	4.17	0.14	3.15	0.08	3.53	0.34	4.52	0.55
245	4.06	0.30	3.94	0.10	3.78	0.07	4.23	0.14	3.52	0.01

STD – standard deviation;
n.d. – not detected (below the detection limit; < 1 log CFU/mL for brine and < 1.4 log CFU/g for table olives).

Table A3. Complete microbial (*Enterobacteriaceae*) monitoring results for brine and *Cobrançosa* olives fermented under plan A.

Fermentation time (days)	Trial									
	AC		A0		A3		A5		A7	
	Mean	STD	Mean	STD	Mean	STD	Mean	STD	Mean	STD
Brine (log CFU/mL)										
0	3.86	0.00	1.93	2.73	3.86	0.00	3.86	0.00	3.86	0.00
7	5.68	0.00	5.84	0.21	6.75	0.00	6.63	0.00	6.00	0.00
14	4.58	0.00	6.00	0.00	6.00	0.00	4.75	0.00	6.00	0.00
21	4.02	0.00	2.00	0.31	4.76	0.00	4.21	0.00	3.66	0.00
28	2.74	0.00	n.d.		2.52	0.09	3.10	0.00	2.40	0.00
35	2.00	0.00	n.d.		n.d.		4.39	0.00	2.00	0.00
56	4.58	0.00	n.d.		n.d.		5.72	0.00	6.14	0.00
63	3.00	0.00	n.d.		n.d.		6.00	0.00	3.00	0.00
77	n.d.		n.d.		n.d.		7.00	0.00	2.48	0.00
98	n.d.		n.d.		n.d.		n.d.		n.d.	
119	n.d.		n.d.		n.d.		n.d.		n.d.	
126	n.d.		n.d.		n.d.		n.d.		n.d.	
161	n.d.		n.d.		n.d.		n.d.		n.d.	
245	n.d.		n.d.		n.d.		n.d.		n.d.	
Table olives (log CFU/g)										
0	3.65	0.00	3.65	0.00	3.65	0.00	3.65	0.00	3.65	0.00
7	4.78	0.30	4.84	0.69	4.55	0.13	4.50	0.31	4.29	0.20
14	2.84	0.20	1.98	0.61	3.09	0.34	3.15	0.02	1.40	0.75
21	1.40	0.04	n.d.		n.d.		1.83	0.61	1.40	0.04
28	1.40	0.40	n.d.		n.d.		1.40	0.28	1.40	0.40
35	3.13	0.59	n.d.		n.d.		1.82	0.12	4.25	0.25
56	4.52	1.42	n.d.		n.d.		4.81	0.27	5.39	0.35
63	n.d.		n.d.		n.d.		2.62	0.54	n.d.	
77	n.d.		n.d.		n.d.		5.09	0.27	n.d.	
98	n.d.		n.d.		n.d.		n.d.		n.d.	
119	n.d.		n.d.		n.d.		n.d.		n.d.	
126	n.d.		n.d.		n.d.		n.d.		n.d.	
161	n.d.		n.d.		n.d.		n.d.		n.d.	
245	n.d.		n.d.		n.d.		n.d.		n.d.	

STD – standard deviation;
n.d. – not detected (below the detection limit; < 1 log CFU/mL for brine and < 1.4 log CFU/g for table olives).

Table A4. Complete microbial (LAB) monitoring results for brine and *Cobrançosa* olives fermented under plan B.

Fermentation time (days)	Trial					
	BC		B5		B7	
	Mean	STD	Mean	STD	Mean	STD
Brine (log CFU/mL)						
0	n.d.		n.d.		n.d.	
140	n.d.		n.d.		n.d.	
147	n.d.		6.94	0.13	n.d.	
161	n.d.		7.94	0.11	n.d.	
182	n.d.		7.52	0.04	n.d.	
189	n.d.		7.78	0.15	n.d.	
203	n.d.		7.85	0.11	n.d.	
210	n.d.		7.86	0.01	1.45	0.21
224	n.d.		7.69	0.13	1.15	0.64
245	3.13	0.04	7.52	0.13	4.66	0.23
Table olives (log CFU/g)						
0	n.d.		n.d.		n.d.	
140	n.d.		n.d.		n.d.	
147	n.d.		4.84	0.19	n.d.	
161	n.d.		5.62	0.01	n.d.	
182	n.d.		5.81	0.28	n.d.	
189	n.d.		5.79	0.23	n.d.	
203	n.d.		5.81	0.03	n.d.	
210	n.d.		5.79	0.22	n.d.	
224	n.d.		5.98	0.21	1.40	0.00
245	n.d.		6.02	0.11	2.42	0.01

STD – standard deviation;
n.d. – not detected (below the detection limit; < 1 log CFU/mL for brine and < 1.4 log CFU/g for table olives).

Table A5. Complete microbial (yeasts) monitoring results for brine and *Cobrançosa* olives fermented under plan B.

Fermentation time (days)	Trial					
	BC		B5		B7	
	Mean	STD	Mean	STD	Mean	STD
Brine (log CFU/mL)						
0	5.28	0.03	5.39	0.02	5.33	0.01
140	5.03	0.00	5.03	0.00	5.03	0.00
147	4.87	0.07	5.88	0.14	4.87	0.07
161	6.16	0.04	6.75	0.35	6.16	0.04
182	6.42	0.48	6.97	0.12	6.02	0.04
189	6.24	0.16	6.10	0.08	5.80	0.29
203	6.20	0.36	6.50	0.16	6.29	0.11
210	6.42	0.05	6.82	0.12	6.88	0.36
224	6.24	0.12	6.53	0.11	6.31	0.04
245	6.35	0.02	6.61	0.01	6.90	0.07
Table olives (log CFU/g)						
0	3.89	0.02	4.15	0.07	3.87	0.02
140	4.03	0.00	4.03	0.00	4.03	0.00
147	4.20	0.04	4.39	0.06	4.20	0.04
161	4.02	0.02	4.80	0.48	4.02	0.02
182	4.81	0.05	4.69	0.03	4.79	0.01
189	4.53	0.25	4.82	0.03	4.40	0.74
203	4.19	0.20	4.32	0.08	4.42	0.36
210	4.39	0.02	4.23	0.08	4.22	0.17
224	4.03	0.21	4.08	0.18	4.04	0.11
245	4.02	0.01	3.96	0.52	4.11	0.18

STD – standard deviation;
n.d. – not detected (below the detection limit; < 1 log CFU/mL for brine and < 1.4 log CFU/g for table olives).

Table A6. Complete microbial (LAB) monitoring results for brine and *Cobrançosa* olives fermented under plan C.

Fermentation time (days)	Trial			
	CC		C5	
	Mean	STD	Mean	STD
Brine (log CFU/mL)				
0	n.d.		n.d.	
7	n.d.		7.08	0.02
17	n.d.		6.76	0.19
21	n.d.		6.88	0.36
28	n.d.		7.26	0.07
42	n.d.		7.19	0.03
64	1.34	0.89	7.61	0.09
Table olives (log CFU/g)				
0	n.d.		n.d.	
7	n.d.		4.34	0.02
17	n.d.		5.05	0.36
21	n.d.		5.38	0.16
28	n.d.		6.07	0.02
42	n.d.		6.38	0.06
64	2.52	0.40	6.23	0.10

STD – standard deviation;
n.d. – no detected (below the detection limit; < 1 log CFU/mL for brine and < 1.4 log CFU/g for table olives).

Table A7. Complete microbial (yeasts) monitoring results for brine and *Cobrançosa* olives fermented under plan C.

Fermentation time (days)	Trial			
	CC		C5	
	Mean	STD	Mean	STD
Brine (log CFU/mL)				
0	5.96	0.37	5.96	0.17
7	5.78	0.04	5.92	0.06
17	6.13	0.04	6.94	0.10
21	6.21	0.06	6.58	0.00
28	6.90	0.27	7.11	0.03
42	6.16	0.05	6.83	0.02
64	7.07	0.22	7.02	0.05
Table olives (log CFU/g)				
0	3.89	0.30	3.79	0.01
7	3.69	0.05	2.94	0.19
17	4.04	0.03	4.85	0.04
21	4.26	0.27	4.60	0.14
28	4.66	0.11	4.88	0.07
42	4.18	0.10	4.69	0.08
64	5.56	0.05	5.61	0.17

STD – standard deviation;
n.d. – no detected (below the detection limit; < 1 log CFU/mL for brine and < 1.4 log CFU/g for table olives).

III. Physicochemical analysis – salt, pH and TTA

Table A8. Complete physicochemical properties results (salt content, pH and total titratable acidity) of brine throughout fermentation of plan A.

Trial		Fermentation time (days)													
		0	7	14	21	28	35	56	63	77	98	119	126	161	245
Salt Content (%)															
AC	**Mean**	0.00	0.00	0.00	3.00	2.90	3.00	3.75	5.00	4.70	4.85	5.00	6.70	5.70	5.50
	STD	0.00	0.00	0.00	0.00	0.14	0.00	0.35	0.00	0.00	0.07	0.00	0.28	0.14	0.00
A0	**Mean**	0.00	0.00	0.00	3.00	3.80	4.20	4.50	5.00	5.00	5.15	5.50	6.75	5.90	5.80
	STD	0.00	0.00	0.00	0.00	0.28	0.00	0.00	0.00	0.00	0.21	0.00	0.35	0.14	0.00
A3	**Mean**	0.00	0.00	0.00	3.00	3.00	3.00	3.50	5.00	4.75	4.95	5.50	6.25	5.75	5.65
	STD	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.07	0.07	0.00	0.35	0.35	0.07
A5	**Mean**	0.00	0.00	0.00	3.00	2.85	3.00	3.50	5.00	4.55	4.50	5.00	6.00	5.60	5.95
	STD	0.00	0.00	0.00	0.00	0.07	0.00	0.00	0.00	0.07	0.00	0.00	0.00	0.00	0.21
A7	**Mean**	0.00	0.00	0.00	3.00	3.00	3.00	3.50	4.90	4.55	4.70	5.20	6.80	5.55	5.50
	STD	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.14	0.07	0.14	0.28	0.28	0.07	0.00
pH															
AC	**Mean**	5.20	4.71	4.56	4.62	4.40	4.41	4.42	4.44	4.37	4.18	4.25	4.20	4.54	4.56
	STD	0.00	0.01	0.03	0.07	0.03	0.08	0.05	0.03	0.05	0.01	0.07	0.07	0.21	0.06
A0	**Mean**	5.20	4.73	4.93	3.99	3.83	3.83	4.00	3.95	4.05	4.28	4.35	4.22	4.32	4.65
	STD	0.00	0.00	0.02	0.12	0.01	0.02	0.08	0.04	0.07	0.00	0.05	0.06	0.06	0.13
A3	**Mean**	5.20	4.70	4.62	4.66	3.87	3.61	3.64	3.61	3.71	4.17	4.17	4.09	4.30	4.83
	STD	0.00	0.00	0.01	0.06	0.11	0.03	0.06	0.03	0.03	0.10	0.11	0.09	0.06	0.09
A5	**Mean**	5.20	4.65	4.60	4.67	4.40	4.37	4.45	4.54	4.70	4.31	4.16	4.08	4.61	5.08
	STD	0.00	0.00	0.05	0.02	0.03	0.03	0.08	0.03	0.07	0.11	0.02	0.02	0.28	0.16
A7	**Mean**	5.20	4.68	4.60	4.67	4.42	4.46	4.56	4.60	4.49	4.36	4.35	4.39	5.00	5.19
	STD	0.00	0.02	0.01	0.00	0.02	0.03	0.03	0.01	0.07	0.04	0.11	0.08	0.71	0.14
Total titratable acidity (%)															
AC	**Mean**	0.15	0.15	0.18	0.16	0.20	0.14	0.24	0.23	0.27	0.50	0.43	0.40	0.22	0.23
	STD	0.04	0.04	0.01	0.03	0.02	0.03	0.03	0.03	0.02	0.06	0.03	0.06	0.06	0.01
A0	**Mean**	0.16	0.16	0.40	0.52	0.56	0.55	0.52	0.54	0.45	0.36	0.43	0.46	0.41	0.27
	STD	0.01	0.01	0.00	0.10	0.01	0.01	0.10	0.03	0.03	0.00	0.06	0.04	0.07	0.04
A3	**Mean**	0.17	0.17	0.18	0.16	0.19	0.39	0.55	0.59	0.61	0.38	0.37	0.42	0.28	0.19
	STD	0.02	0.02	0.00	0.03	0.01	0.02	0.04	0.01	0.04	0.03	0.01	0.04	0.01	0.03
A5	**Mean**	0.15	0.15	0.16	0.15	0.11	0.16	0.24	0.20	0.33	0.37	0.47	0.51	0.26	0.17
	STD	0.01	0.01	0.01	0.01	0.00	0.00	0.03	0.02	0.07	0.14	0.02	0.01	0.07	0.03
A7	**Mean**	0.18	0.18	0.20	0.16	0.09	0.18	0.21	0.18	0.29	0.28	0.39	0.27	0.24	0.14
	STD	0.01	0.01	0.00	0.01	0.00	0.07	0.00	0.03	0.04	0.09	0.03	0.03	0.04	0.01

STD – standard deviation.

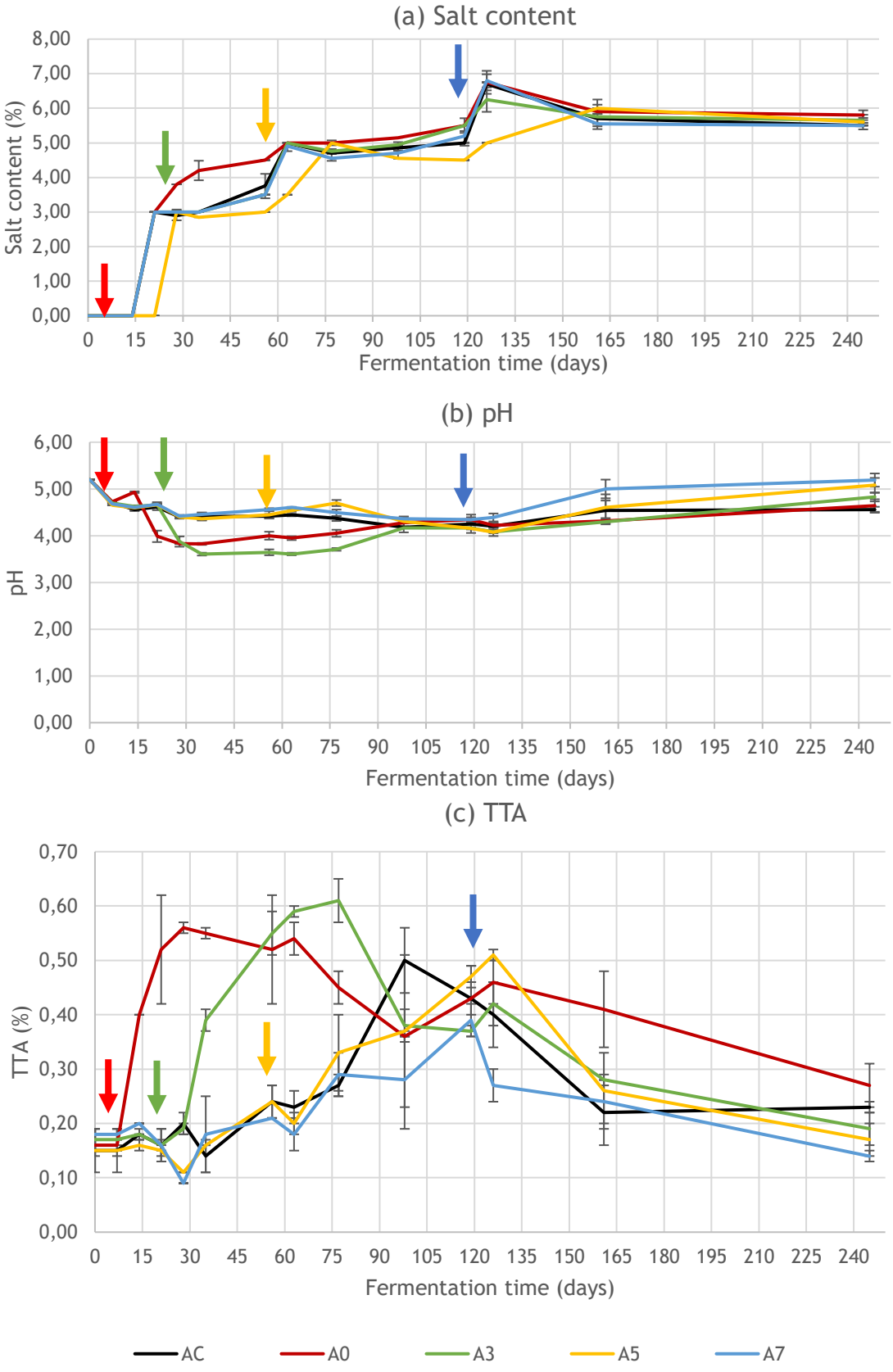

Figure A2. Physicochemical properties: (a) salt content, (b) pH and (c) total titratable acidity (TTA). of brine throughout fermentation of plan A. Arrows correspond to, the moment of inoculation with *Lpb. pentosus* i106 to the experimental trial, and the corresponding salt addition to all vessels.

Table A9. Complete physicochemical properties results (salt content. pH and total titratable acidity) of brine throughout fermentation of plan B.

Trial		Fermentation time (days)									
		0	140	147	161	182	189	203	210	224	245
Salt Content (%)											
BC	**Mean**	4.35	3.75	3.20	3.20	6.50	5.95	6.00	6.00	5.90	4.35
	STD	0.21	0.07	0.00	0.14	0.00	0.07	0.00	0.00	0.14	0.21
B5	**Mean**	4.35	4.25	3.50	3.40	6.20	5.45	5.65	5.55	5.55	4.35
	STD	0.21	0.07	0.00	0.00	0.28	0.07	0.21	0.07	0.07	0.21
B7	**Mean**	4.35	3.75	3.20	3.05	6.50	5.60	5.85	5.60	5.60	4.35
	STD	0.21	0.07	0.00	0.07	0.00	0.00	0.07	0.00	0.00	0.21
pH											
BC	**Mean**	4.17	3.98	4.03	4.14	4.01	4.06	4.09	4.17	4.17	4.17
	STD	0.05	0.02	0.00	0.03	0.01	0.09	0.06	0.02	0.04	0.05
B5	**Mean**	4.19	3.76	4.88	4.63	4.24	4.85	4.76	4.67	4.83	4.19
	STD	0.01	0.02	0.03	0.03	0.02	0.01	0.05	0.04	0.08	0.01
B7	**Mean**	4.18	3.98	4.02	4.11	4.00	4.51	4.53	4.54	4.71	4.18
	STD	0.04	0.02	0.03	0.05	0.07	0.08	0.06	0.02	0.04	0.04
Total titratable acidity (%)											
BC	**Mean**	0.05	0.21	0.21	0.21	0.12	0.11	0.11	0.09	0.11	0.05
	STD	0.00	0.05	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.00
B5	**Mean**	0.05	0.16	0.11	0.11	0.15	0.07	0.06	0.08	0.07	0.05
	STD	0.00	0.02	0.00	0.01	0.00	0.01	0.00	0.01	0.01	0.00
B7	**Mean**	0.05	0.14	0.11	0.13	0.07	0.08	0.05	0.07	0.07	0.05
	STD	0.00	0.02	0.00	0.02	0.02	0.02	0.00	0.02	0.01	0.00

STD – standard deviation.

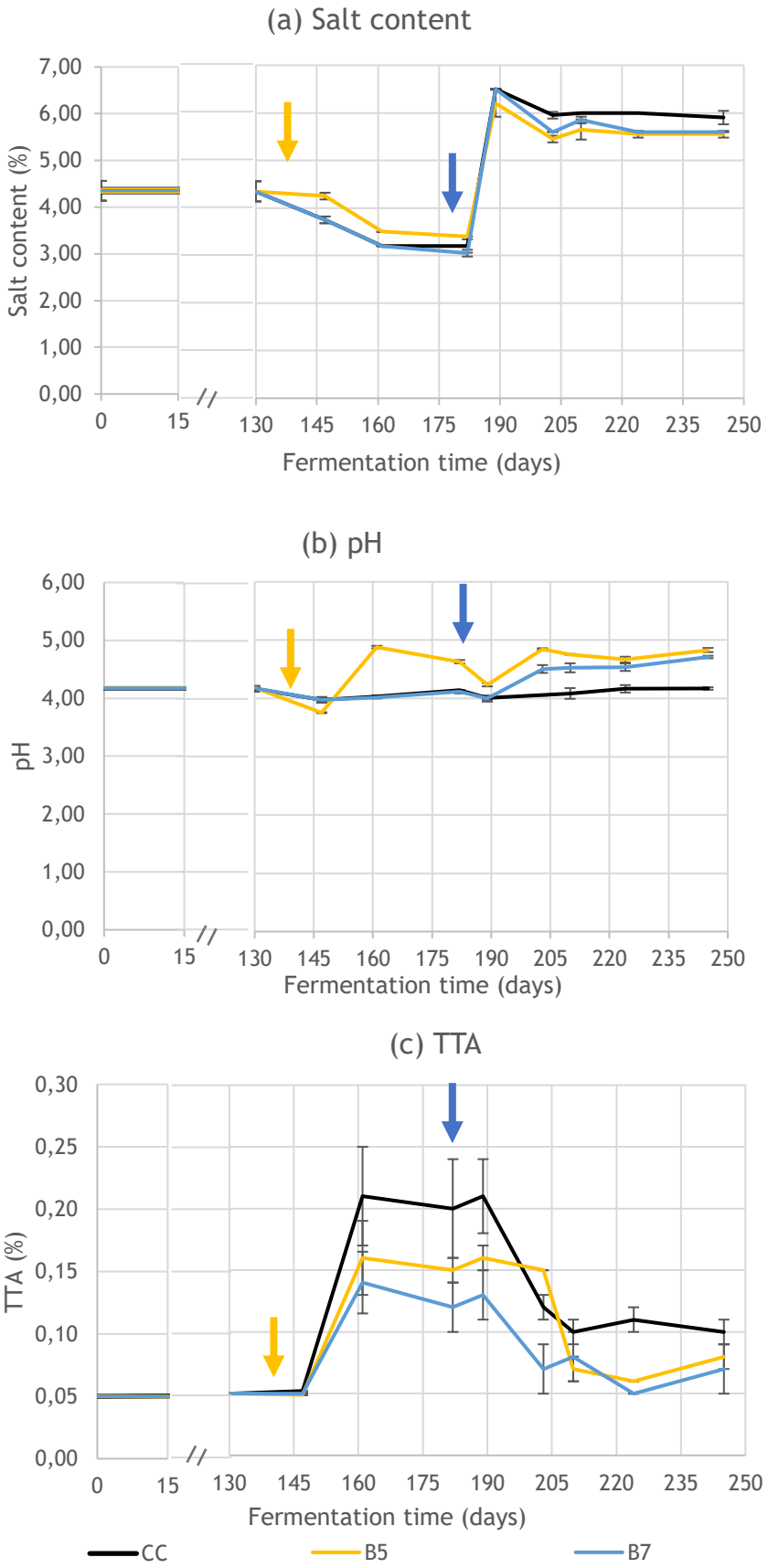

Figure A3. Physicochemical properties: (a) salt content, (b) pH, and (c) total titratable acidity of brine throughout fermentation of plan B. Arrows correspond to the moment of inoculation with *Lpb. pentosus* i106 to the experimental trial, and the corresponding salt addition to all vessels.

Table A10. Complete physicochemical properties results: salt content, pH and total titratable acidity of brine throughout fermentation of plan C.

Trial		Fermentation time (days)						
		0	7	17	21	28	42	64
Salt Content (%)								
CC	Mean	5.10	5.75	5.70	5.75	5.55	5.55	5.75
	STD	0.14	0.35	0.00	0.35	0.07	0.07	0.21
C5	Mean	5.00	6.00	6.00	5.70	5.85	5.85	5.75
	STD	0.00	0.00	0.00	0.14	0.07	0.07	0.07
pH								
CC	Mean	4.03	4.04	4.09	4.16	4.25	4.22	4.40
	STD	0.04	0.01	0.01	0.00	0.00	0.01	0.08
C5	Mean	4.10	3.58	3.72	3.91	4.13	4.24	4.46
	STD	0.03	0.01	0.03	0.05	0.10	0.01	0.09
Total titratable acidity (%)								
CC	Mean	0.14	0.14	0.13	0.11	0.11	0.12	0.10
	STD	0.03	0.02	0.01	0.00	0.01	0.01	0.03
C5	Mean	0.14	0.29	0.21	0.16	0.14	0.13	0.07
	STD	0.01	0.01	0.02	0.01	0.01	0.01	0.01

STD – standard deviation.

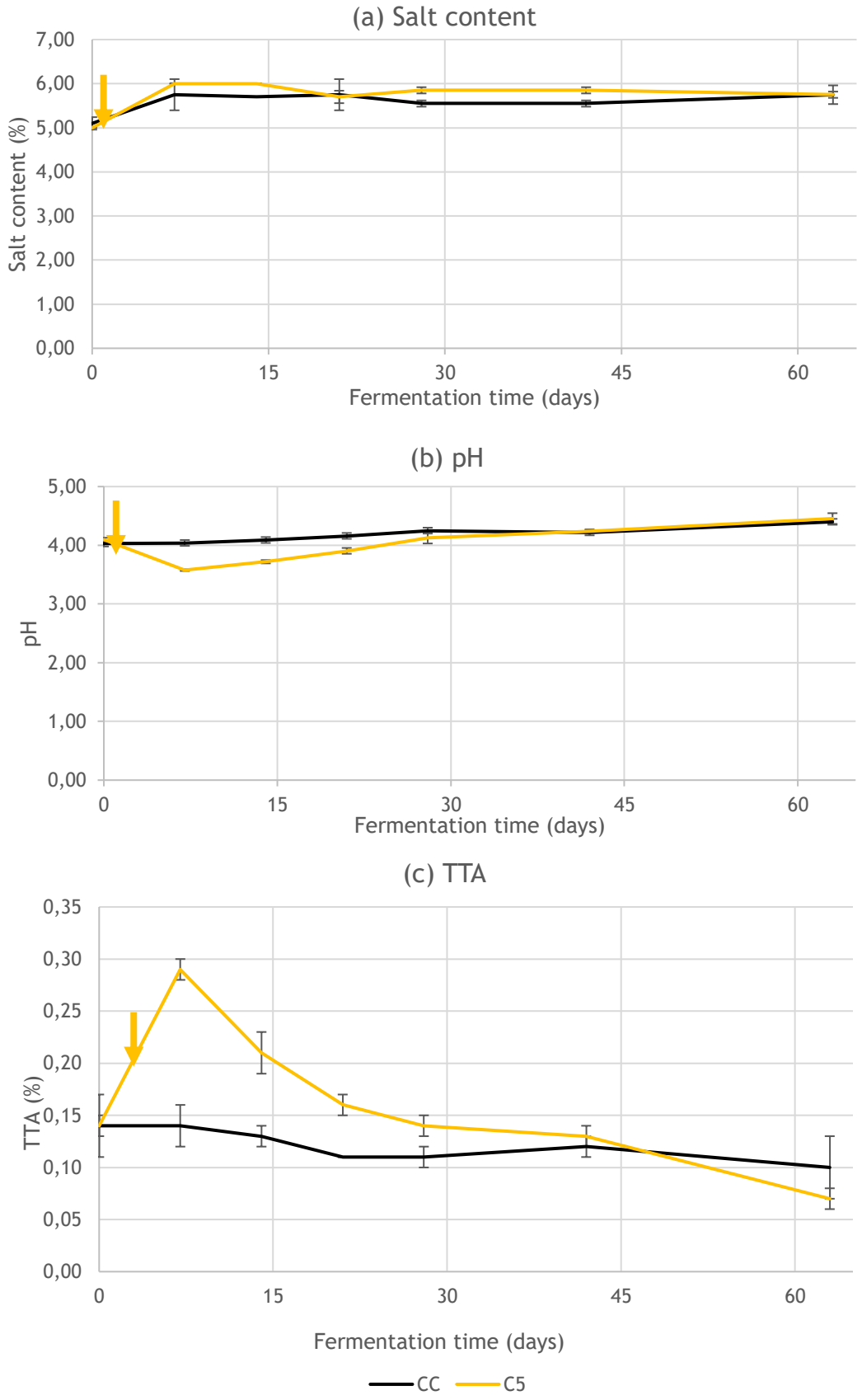

Figure A4. Physicochemical properties: (a) salt content, (b) pH, and (c) total titratable acidity of brine throughout fermentation of plan C. Arrows correspond to the moment of inoculation with *Lpb. pentosus* i106 to the experimental trial, and the corresponding salt addition to all vessels.

IV. Physicochemical analysis - phenolic compounds

Considering all phenolic compounds analysed (oleuropein, hydroxytyrosol, tyrosol, verbascoside - **Figure A5** and **Figure A6**) and the internal standard (syringic acid) for concentration calculation, the regression curves presented in **Table A11** were used, as well as the LOD (detection limit) and LOQ (limit of quantitation) of the compounds at 280 nm.

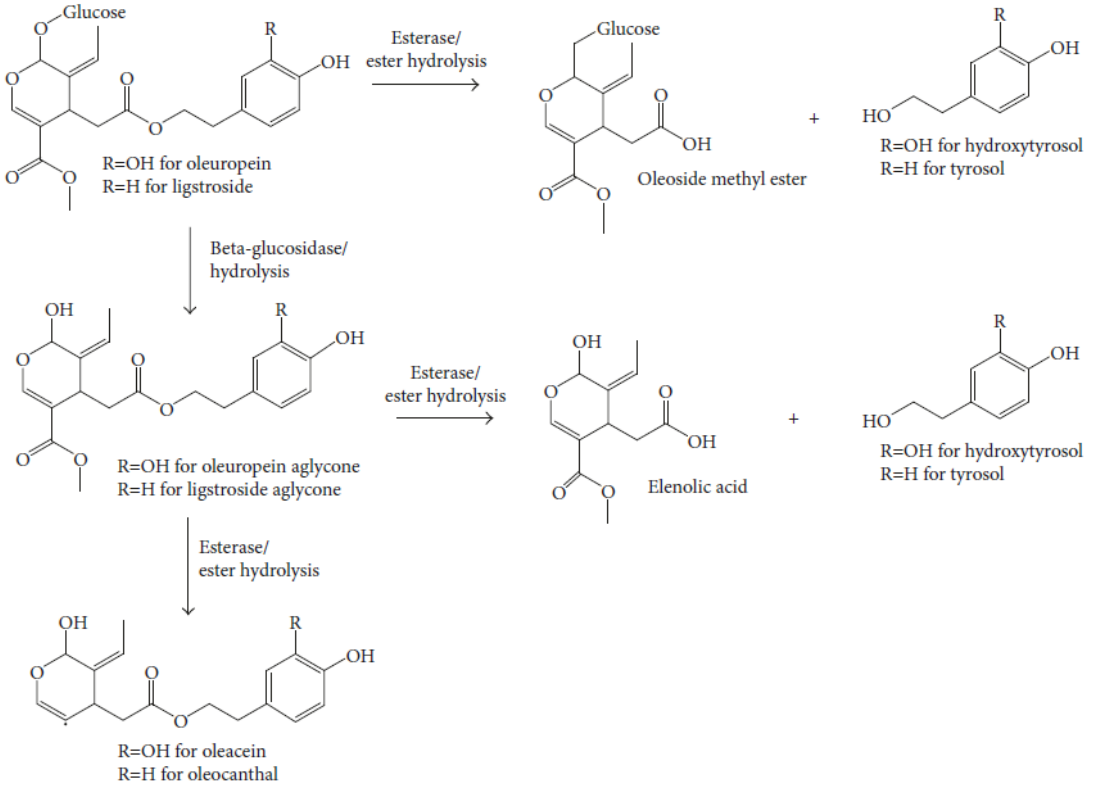

Figure A5. Oleuropein and ligstroside hydrolysis by esterase and β-glucosidase activity to hydroxytyrosol and tyrosol (Johnson and Mitchell, 2018).

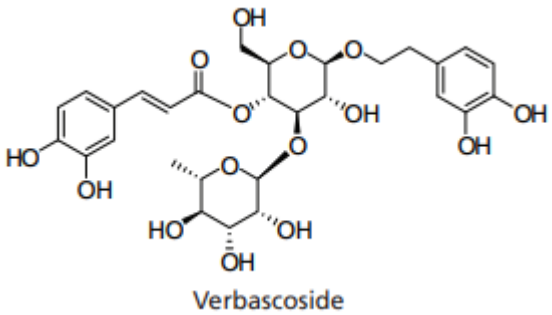

Figure A6. Verbascoside chemical structure (Termentzi *et al.*, 2015).

Table A11. Calibration curves and detection limits (LOD. LOQ) of phenolic compounds (oleuropein. hydroxytyrosol. tyrosol. verbascoside) and internal standard (syringic acid) concentrations at 280 nm.

Compound	Regression curve	R^2	LOD	LOQ
Oleuropein	$y = (4.57 \times 10^6)x + (4.49 \times 10^4)$	0.9996	0.042	0.127
Hydroxytyrosol	$y = (1.85 \times 10^7)x + (6.88 \times 10^5)$	0.9938	0.140	0.424
Tyrosol	$y = (9.94 \times 10^6)x + (3.20 \times 10^5)$	0.9951	0.121	0.366
Verbascoside	$y = (1.60 \times 10^7)x + (1.06 \times 10^5)$	0.9994	0.022	0.067
Siryngic acid	$y = (9.67 \times 10^7)x + (1.58 \times 10^5)$	0.9982	0.006	0.019

Note: x = OD_{280}

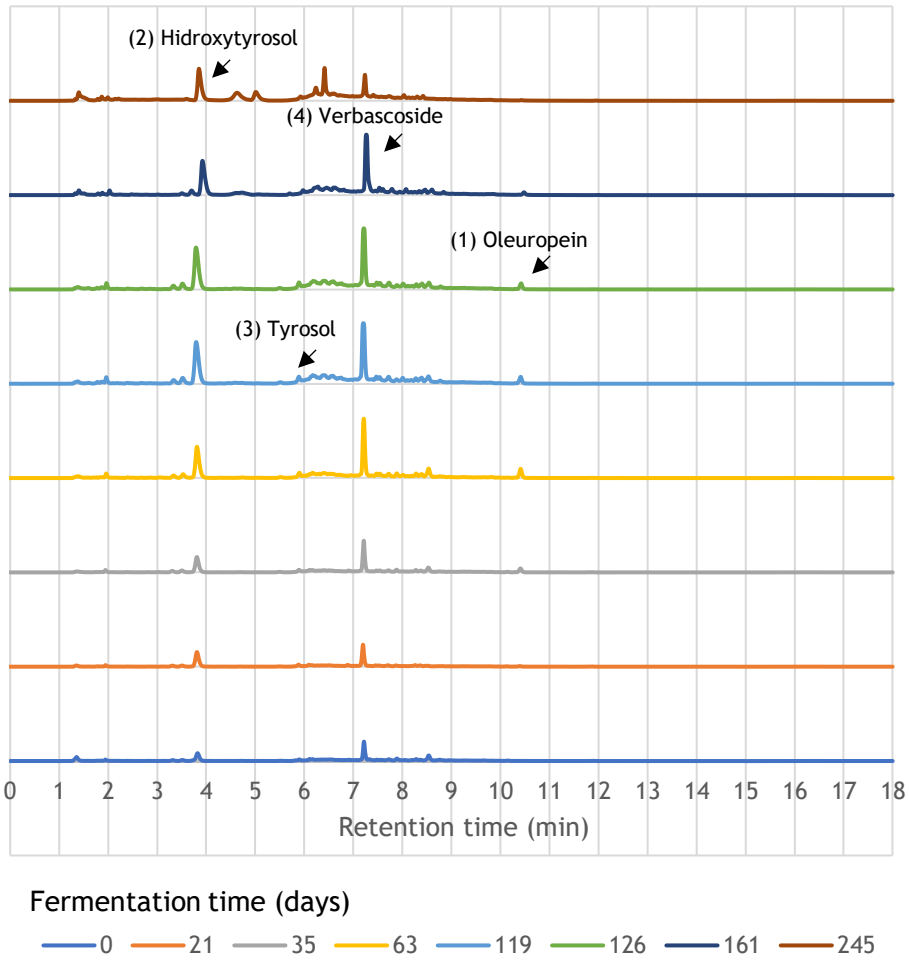

Figure A7. Example of chromatograms obtained at throughout the fermentation for plan A; Peaks: (1) oleuropein; (2) hydroxytyrosol; (3) tyrosol; (4) verbascoside.

Table A12. Complete phenolic profile results of table olive brine throughout fermentation of plan A - oleuropein, hydroxytyrosol, tyrosol, and verbascoside.

Trial		Fermentation time (days)							
		0	14	28	63	119	126	161	245
Oleuropein (mg/L)									
AC	**Mean**	343.1	54.0	298.3	612.0	552.5	545.0	392.8	nd
	STD	60.6	9.2	41.9	75.2	2.1	35.9	64.1	
A0	**Mean**	338.3	636.3	876.7	861.4	699.9	636.3	431.0	nd
	STD	35.9	36.3	18.7	29.1	35.0	35.7	34.4	
A3	**Mean**	301.1	72.0	269.5	521.3	509.0	466.0	360.0	nd
	STD	41.8	10.4	6.0	20.8	127.3	101.2	25.6	
A5	**Mean**	295.7	48.6	209.9	412.9	361.1	376.1	354.8	nd
	STD	49.1	3.2	12.9	53.9	6.4	25.8	20.1	
A7	**Mean**	351.7	46.8	201.5	322.0	320.0	300.8	238.3	nd
	STD	1.6	5.5	16.6	53.7	44.3	26.0	27.3	
Hydroxytyrosol (mg/L)									
AC	**Mean**	nd	208.8	200.6	483.7	834.5	833.6	655.1	469.9
	STD		30.3	7.7	15.7	139.8	137.2	129.4	34.1
A0	**Mean**	nd	389.0	553.5	818.1	1118.4	1126.7	996.5	594.2
	STD		23.9	8.4	7.4	54.7	56.4	57.5	97.1
A3	**Mean**	nd	238.1	275.3	611.3	858.9	866.5	633.5	318.0
	STD		36.2	5.4	26.1	4.5	23.4	35.1	33.1
A5	**Mean**	nd	197.8	213.3	480.0	950.7	964.8	543.8	173.4
	STD		11.9	12.9	7.1	15.6	13.2	96.9	31.3
A7	**Mean**	nd	206.3	190.6	467.9	785.9	608.4	434.4	162.7
	STD		29.4	16.9	18.0	21.8	3.2	9.1	2.7
Tyrosol (mg/L)									
AC	**Mean**	nd	nd	nd	132.1	215.0	203.7	171.9	nd
	STD				14.6	42.7	33.1	26.8	
A0	**Mean**	nd	nd	155.4	202.4	242.6	266.2	nd	nd
	STD			20.8	2.8	3.9	41.1		
A3	**Mean**	nd	nd	nd	135.6	164.6	164.5	nd	nd
	STD				4.5	2.1	10.8		
A5	**Mean**	nd	nd	nd	131.7	197.7	186.6	nd	nd
	STD				19.3	11.7	7.9		
A7	**Mean**	nd	nd	nd	n.d.	189.7	198.6	nd	nd
	STD					8.8	6.3		
Verbascoside (mg/L)									
AC	**Mean**	214.0	293.0	322.2	701.2	832.1	915.2	714.7	nd
	STD	10.6	50.6	38.9	49.5	100.0	17.9	129.6	
A0	**Mean**	224.0	515.3	673.9	836.1	901.1	655.6	356.4	nd
	STD	6.7	10.1	44.2	13.5	100.0	19.8	12.8	
A3	**Mean**	215.9	283.5	362.5	637.2	880.4	929.1	425.1	nd
	STD	23.6	8.9	10.9	9.5	2.7	54.9	49.6	
A5	**Mean**	211.2	266.7	374.3	691.7	820.5	715.9	457.5	nd
	STD	19.5	8.1	3.4	66.4	20.3	11.8	10.5	
A7	**Mean**	231.7	268.5	326.3	693.2	939.9	756.4	582.0	nd
	STD	26.1	11.3	48.7	82.8	34.6	12.1	107.7	

STD – standard deviation; nd – not determined (below the detection limit)

Table A13. Complete phenolic profile results of table olive brine throughout fermentation of plan B - oleuropein, hydroxytyrosol, tyrosol and verbascoside.

Trial		Fermentation time (days)							
		0	**140**	**182**	**189**	**203**	**210**	**224**	**245**
Oleuropein (mg/L)									
BC	**Mean**	nd	63.4	108.4	120.3	130.1	150.0	172.2	159.8
	STD		3.0	21.1	14.5	19.3	31.3	19.7	31.7
B5	**Mean**	50.7	58.8	42.0	47.7	72.0	67.7	53.6	70.0
	STD	0.5	11.7	6.1	6.4	6.3	0.3	10.5	14.9
B7	**Mean**	50.7	61.1	122.5	107.6	127.4	132.2	85.7	43.0
	STD	0.5	0.5	0.8	16.9	4.8	2.4	17.8	0.6
Hydroxytyrosol (mg/L)									
BC	**Mean**	nd	nd	154.3	159.8	170.5	136.6	139.9	nd
	STD			30.1	22.8	32.4	25.4	19.4	
B5	**Mean**	nd	nd	139.9	139.9	139.9	139.9	143.7	nd
	STD			4.6	3.4	21.5	31.9	23.9	
B7	**Mean**	nd	nd	214.8	210.0	228.6	246.0	242.2	243.9
	STD			0.3	23.3	10.2	13.1	22.3	19.1
Tyrosol (mg/L)									
BC	**Mean**	nd	nd	nd	nd	nd	nd	nd	nd
	STD								
B5	**Mean**	nd	nd	nd	nd	nd	nd	nd	nd
	STD								
B7	**Mean**	nd	nd	nd	nd	nd	nd	nd	nd
	STD								
Verbascoside (mg/L)									
BC	**Mean**	53.4	153.1	270.5	266.7	363.3	393.0	400.7	441.2
	STD	0.4	5.9	11.6	25.0	4.8	17.8	0.4	18.8
B5	**Mean**	49.3	142.0	230.1	204.5	272.7	297.4	317.1	304.6
	STD	1.8	0.0	10.4	0.1	13.8	12.3	7.1	54.1
B7	**Mean**	49.3	139.1	279.8	249.9	342.9	368.4	402.2	457.6
	STD	1.8	12.3	16.9	29.6	16.5	18.3	22.0	28.9

STD – standard deviation; nd – not determined (below the detection limit)

Table A14. Complete phenolic profile results of table olives' brine throughout fermentation of plan C - oleuropein, hydroxytyrosol, tyrosol and verbascoside.

Trial		Fermentation time (days)						
		0	7	14	21	28	42	64
Oleuropein (mg/L)								
CC	**Mean**	105.5	95.9	109.5	121.7	129.9	119.4	149.9
	STD	20.3	2.0	2.9	1.8	3.0	15.0	7.3
C5	**Mean**	85.3	54.6	57.9	63.7	67.7	71.0	72.7
	STD	19.9	10.0	12.0	12.6	12.9	5.8	1.9
Hydroxytyrosol (mg/L)								
CC	**Mean**	290.9	322.4	367.3	392.0	371.3	285.3	186.0
	STD	34.7	14.5	5.3	3.1	4.5	2.6	33.2
C5	**Mean**	271.6	265.5	295.7	331.8	356.7	318.9	267.5
	STD	31.7	8.9	17.8	11.2	15.1	5.2	3.6
Tyrosol (mg/L)								
CC	**nd**	nd	nd	nd	nd	nd	nd	nd
C5	**nd**	nd	nd	nd	nd	nd	nd	nd
Verbascoside (mg/L)								
CC	**Mean**	46.5	345.0	376.8	413.1	440.0	452.1	488.2
	STD	8.8	9.6	14.1	8.0	16.6	18.9	49.5
C5	**Mean**	44.2	225.6	249.3	272.9	287.7	210.1	258.3
	STD	3.8	35.0	40.5	45.0	50.4	36.8	0.0

STD – standard deviation; nd – not determined (below the detection limit)

V. Sensory analysis

In this section, an example of a questionnaire provided to the panellists in order to obtain the sensory analysis results of **Table A15** is presented**.**

Primeiro e último NOME: _____

CÓDIGO DA AMOSTRA: _____

Colocar um X NA RECTA onde achar apropriado:

PARÂMETRO	INTENSIDADE		
Textura	Mole	_____	Coerente
Sabor	Ausente	_____	Forte
Salgado	Não	_____	Muito
Amargor	Não	_____	Alto
Acidez	Não	_____	Alta
Sabor anormal	Ausente	_____	Forte
Avaliação global	Não gosto nada	_____	Gosto muito

Outros defeitos? Não _____ **Sim**_____

Especificar: coloca um X onde achar apropriado

Bolorento		**Metálico**	
Rançoso		**Terroso**	
Cozinhado		**Avinagrado**	
Saponificado		**Podridão**	

Outros defeitos – especificar:

Table A15. Complete results of sensory assessment – texture, flavour, saltiness, bitterness, acidity, abnormal flavours, overall eating quality, of *Cobrançosa* table olives fermented under three different plans (A, B and C). Mean values are presented within a scale from 1 to 10.

	Trial									
	Plan A					Plan B			Plan C	
	AC	A0	A3	A5	A7	BC	B5	B7	CC	C5
Texture										
Mean	4.16	4.34	2.58	4.44	1.58	5.44	4.78	5.38	7.89	6.52
STD	2.46	2.09	1.64	2.86	0.75	2.75	2.58	2.39	1.75	2.76
CVr (%)	19.9	18.0	19.0	17.5	24.3	18.4	20.0	18.2	3.30	14.0
Flavour										
Mean	5.48	5.63	5.49	5.59	4.00	5.63	4.53	5.72	5.88	5.82
STD	2.39	1.74	2.46	2.82	2.12	2.18	2.08	2.60	2.71	2.15
CVr (%)	15.8	12.0	19.4	17.9	15.7	19.5	22.8	20.7	17.1	8.80
Salty										
Mean	4.34	5.01	4.83	4.24	4.22	4.77	4.57	4.71	4.78	4.18
STD	2.39	2.45	0.94	0.91	1.38	2.64	2.25	1.73	2.38	2.33
CVr (%)	19.1	10.1	3.70	6.10	13.5	11.6	22.0	6.30	16.4	21.6
Bitterness										
Mean	6.14	6.64	5.28	4.36	2.77	5.05	3.14	3.22	2.67	2.84
STD	2.59	2.12	2.95	2.55	1.37	2.30	1.85	1.53	1.31	1.41
CVr (%)	11.6	8.50	15.6	21.7	21.8	8.60	18.9	21.7	22.8	14.7
Acidity										
Mean	4.28	5.21	3.96	3.53	4.01	5.17	2.43	3.90	3.28	2.48
STD	2.63	2.28	1.85	1.82	1.47	2.30	1.37	2.25	1.55	1.13
CVr (%)	22.9	13.6	17.5	20.6	20.0	13.9	17.5	20.0	16.2	18.5
Abnormal flavours										
Mean	4.31	4.69	4.96	4.41	4.52	3.00	3.63	3.21	2.73	3.47
STD	1.66	2.28	2.75	1.38	2.76	1.55	2.09	1.66	1.53	1.42
CVr (%)	17.2	16.1	15.6	14.6	21.5	14.4	22.3	14.8	23.3	24.5
Overall eating quality										
Mean	4.14	4.58	4.27	4.63	3.98	5.41	5.18	5.87	7.43	6.93
STD	1.93	2.17	1.96	2.48	1.73	2.31	2.89	1.85	1.60	2.26
CVr (%)	12.4	18.0	13.2	17.4	19.3	15.8	20.2	7.10	9.00	10.2

STD – standard deviation; CVr – Robust Coefficient of Variation