Faculty of Engineering - University of Porto

Study of Prebiotic Effect of Olive Pomace

Débora Ugulino Araújo

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MASTER ON BIOLOGICAL ENGINEERING

Supervisor: Doctor Josman Dantas Palmeira
Co-supervisor: Professor Doctor Helena Maria Neto Ferreira

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“On ne voit bien qu’avec le coeur. L’essentiel est invisible pour les yeux” -

Saint-Exupéry, Le Petit Prince.

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Abstract

Rapid population growth leads to higher consumption, which, in turn, increases output and waste generation. One of the largest industries producing by-products is the olive oil industry, mainly in the Mediterranean region. The human food sector is interested in the high-added nutritional value foods. Olive pomace (OP), one by-product of olive oil industry, is exceptionally rich in dietary fiber, and phenolic compounds as antioxidants. It needs to be valued with a more mindful and sustainable approach. The purpose of this study was to investigate the possibility of fermenting OP (from Trás-os-Montes) with its own microbiota at different temperatures and analyse its potential as prebiotic.

The OP was subjected to fermentation at room temperature for 32 days. During the process, timepoints were collected at 48 hours, 96 hours, 8 days, 16 days, and 32 days to analyse the microbiological profile over time. The prebiotic activity was analysed using different fermentation timepoints: without fermentation, 48hrs, 96hrs, 32 days of fermentation, with different concentrations of each (2.5%, 5% and 10%). Monoculture suspension of *Lactobacillus fermentum, Lactobacilus paracasei* and *Lactobacillus plantarum* – confirmed probiotics – were prepared and inoculated in Man, Rogosa and Sharpe (MRS) broth with the OP sample. Inulin, glucose, and MRS broth without glucose were used as reference prebiotic, positive control, and negative control, respectively. The sample were then subjected to simulated *in vitro* gastrointestinal digestion, according to INFOGEST protocol, followed by the same prebiotic evaluation assay to confirm the previous results.

In this study, it was possible to verify OP's prebiotic activity in the assay with *L. fermentum*, and an inhibition effect regarding *L. paracasei*. Finally, regarding *L. plantarum*, it was possible to verify an increase in colony-forming units (CFU) per milliliter within OP fermentation time. Through this research, it was possible to verify results that showed a prebiotic potential effect of OP on *L. fermentum* and *L. plantarum*. Further steps of *in vitro* and *in vivo* analysis will be needed to confirm the prebiotic capacity.

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Sincerely,

Débora Araújo.

Summary

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Abbreviation and symbols

ANOVA – Analysis of Variance
BCFA – Big-Chain Fatty Acid
CFU – Colony Forming-Unit
Et al. – And Others
EU – European Union
FAO – Food Agriculture Organization
FOP – Fermented OP
GHG – Green House Gas
GI – Gastrointestinal
MRS – Man, Rogosa and Sharpe
MCFA – Medium-Chain Fatty Acid
MSA – Manitol Salt Agar
OD – Optical Density
OMW – Olive Mill Waste
OMWW – Olive Mill Wastewater
OP – Olive Pomace
PCA – Plate Count Agar
PRF – Polyphenol Rich Fiber
SAB - Sabouraud Dextrose Agar
SCFA – Short Chain Fatty Acid
SGDI – Simulated Gastrointestinal Digestion In Vitro
SGF – Simulated Gastric Fluid
SIF – Simulated Intestinal Fluid
SSF – Simulated Salivary Fluid
T0 – 0 days of fermentation
T2 – 2 days of fermentation
T4 – 4 days of fermentation
T32 – 32 days of fermentation

1. Introduction

Waste and by-product generation have an impact on the environmental, economic, and social sectors. Several biomaterials are not utilized and are deposited in landfills where, due to microbial decomposition, there is an increase in greenhouse gas (GHG) emission and leachate production [1]. The negative economic effects are brought on by the expenses associated with processing solid waste in landfills, since it may be difficult to manage large amounts of various degradable materials [2]. According to estimates from the Food and Agriculture Organization (FAO), nearly a third of all food meant for human consumption is lost or wasted globally.

Nonetheless, one in nine people still lacks adequate nutrition, which equates to almost a billion tons of food and $940 billion in economic losses each year. 40% of food in the US is lost or wasted every year, costing the economy an estimated $218 billion. Food makes up 24% of the solid waste dumped in American landfills, which are the country's third-largest source of methane emissions connected to people. Reducing this food waste offers chances to improve resource and energy conservation, boost productivity and economic efficiency, and combat climate change [3]. Each year, the European Union (EU) produces close to 59 million tons of food waste (131 kg/person), with a market value estimated at 132 billion euros [4]. Around 10% of the food made available to EU customers (at retail, food services, and residences) may be wasted, according to Eurostat's estimations. Meanwhile, 36.2 million individuals nationwide cannot afford a decent dinner every other day [5]. The social impact is due to an ethical and moral dimension within the definition of global food security, since between 702 and 828 million people suffer from hunger [6].

Simultaneously, the population of the planet is increasing. It reached 8 billion on November 15, 2022, and by 2050, it is predicted to reach over 10 billion [7,8]. Therefore, the Earth’s resources are decreasing. Estimates predict that global food demand will increase from 35% to 56% between 2010 and 2050, and the population risk of hunger is expected to change from -91% to +8% in the same period [9]. The upcycling, valorization, and usage of these by-products is a priority, aiming at responsible consumption and production given that the global production of plant-based products is constantly growing and generates enormous waste [10]. The circular economy and residue valorization concepts have come to the forefront to reduce waste, conserve resources, improve production sector efficiency, and value by-products to create value-added products that will help the economy become more sustainable and solve environmental issues. One of the greatest challenges is to valorize residues from the agri-food industry when they still have advantageous nutritional properties.

1.1 Olive oil industry

One of the biggest residue-making industries is the olive oil sector. It leads to the production of by-products (e.g., olive pomace paste and olive leaves) and wastes (e.g., wood and wastewater), representing an important environmental issue in the Mediterranean areas, where they are generated in huge quantities in short periods of time [11]. According to the International Olive Council, about 3,000,000 tons of olive oil are produced every year, but olive oil represents only 20% of the fruit. Consequently, 80% of the olive remains as olive pomace (OP) [12,13]. And more than 12,000,000 tons of this by-product are available each year as an alternative ingredient (after stone removal) that can be valorized [14].

Due to major environmental problems and the associated cost of disposing of olive oil by-products, specifically the OP, there is a stimulus to valorize it [15]. The environmental concern is due to negative physical, chemical, and biological effects on soil; potential phytotoxicity to crops; and potential risk to groundwater [16]. Inhibition of soil microbial activity may in turn reduce soil fertility by inhibiting key processes in nutrient cycling responsible for the formation of labile forms of macro and micro elements; thus, the release of olive mill waste (OMW) into the environment is not recommended [16-18]. Therefore, the possibility of OMW composting has been highlighted in order to transform its chemical profile and turn the phytochemical properties into fertilizer through fermentation [11].

1.2 Olive Pomace

1.2.1 Origin and composition

Olive oil production begins with defoliation and washing, followed by milling, where the oil is extracted from the olive; malaxation, a step that allows the olive oil drops to assemble and facilitate the separation from the aqueous phase; horizontal centrifugation, a step where the by-product olive pomace (OP) and the oil are separated; and vertical centrifugation to remove all the remaining impurities (**Figure 1**) [19].

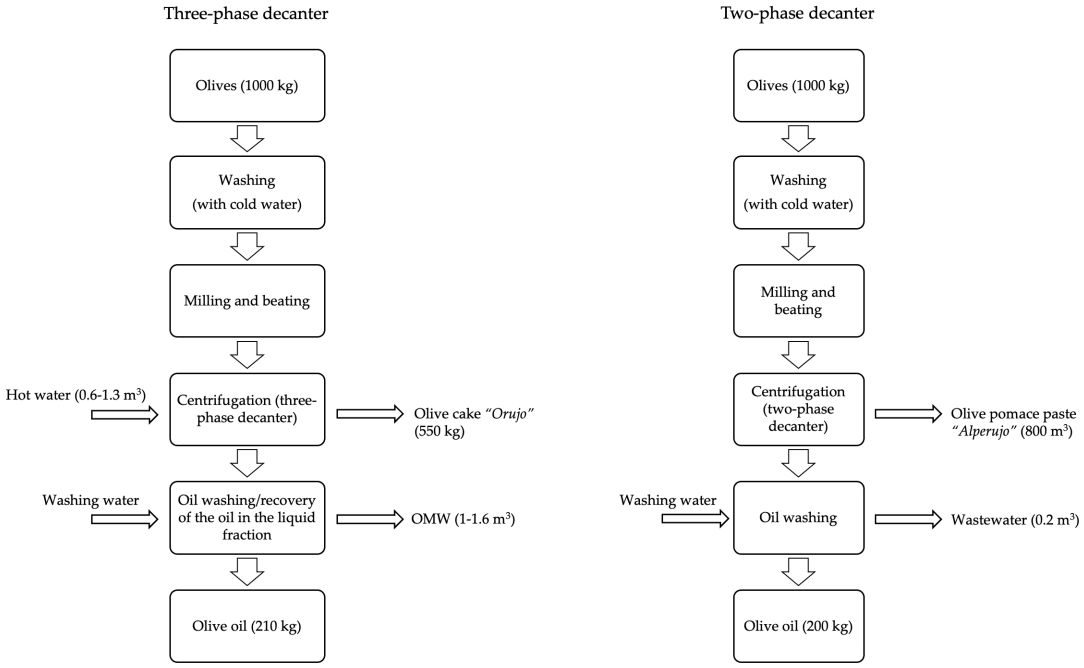

Figure 1: Representation of olive oil extraction, adapted from Alburquerque et al. [20].

The raw OP contains crushed hull, skin, pulp, water, and residual oil [21]. It is composed of small amounts of crude protein and a high percentage of dietary fiber, mainly lignin (27%), followed by cellulose (15%) and hemicellulose (10%) [11]. Nunes *et al*. (2021) verified that an OP extract can be considered an all-in-one advantageous ingredient since it presents a mixture of lipidic and hydrophilic bioactive compounds usually not present in other plant extracts (**Table 1** and **Table 2**). In addition, the authors also observed OP antibacterial activity against gram-positive and gram-negative bacteria [22].

Table 1: Chemical composition of olive pomace [22].

Compounds	Levels
Total fat (g/100g)	4.6-10.5
Fatty acids (relative %)	
C16:0 (Palmitic)	11.6-14.3
C16:1 (Palmitoleic)	0.6-1.3
C17:0 (Heptadecanoic)	0.12-0.19
C18:0 (Stearic)	2.3-3.6
C18:1n9*cis* (Oleic)	71.1-72.9
C18:2n6*cis* (Linoleic)	8.4-10.5
C20:0 (Arachidic)	0.43-0.47
C18:3n3 (α -Linoleic)	0.72-0.9
C20:1n9 (cis-11-Eicosenoic)	0.22-0.3
C22:0 (Behenic)	0.15-0.21

C24:0 (Lignoceic)	0.06-0.08
Total vitamin E (mg/100g)	0.87-2.25
α- Tocopherol	0.77-1.96
α -Tocotrienol	0.04-0.21
β -Tocopherol	0.02-0.05
γ -Tocopherol	0.04-0.07
Total protein (g/100g)	0.9-4.4
Ash (g/100g)	9.9-16.7
pH	5.2-5.6

Table 2: Phenolic compounds present in olive.

Phenolic compounds	References
Phenolic acids	
4-hydroxyphenyl acetic acid	[23]
Caffeic acid	[24-27]
Cinnamic acid	[25,27]
Ferulic acid	[25-27]
Gallic acid	[27]
Homovanillic acid	[26]
p-coumaric acid	[23,26,27]
Sinapic acid	[23,26,27]
Syringic acid	[23,26,27]
Vanillic acid	(26, 27)
Secoiridoids and derivatives	
D3,4-Dihydroxyphenylethanol-elenolic acid dialdehyde	[23,24]
Demethyloleuropein	[24]
Hydroxytyrosol	[28]
Ligstroside	[26]
Oleuropein	[24,28,29]
Tyrosol	[26,28]
Verbascoside	[24,26,29]
Flavonoids	
Apigenin	[28]
Apigenin 7-O-glucoside	(29)
Apigenin 7-O-rutinoside	[28]
Cyanidin 3-O-glucoside	[25,29]
Cyanidin 3-O-rutinoside	[25,29]
Hesperidin	[25,27]
Luteolin	[28]

Luteolin 4'-O-glucoside	[28,30]
Luteolin 7-O-glucoside	[28,29]
Luteolin 7-O-rutinoside	[28]
Quercetin	[27,30]
Quercetin 3-O-glucoside	[28]
Quercetin 3-O-rhamnoside	[26]
Quercetin 3-O-rutinoside	[29]
Rutin	[28-30]

1.2.2 Microbiological profile

The OP microbiota is subsequently influenced by the OP chemical composition, which is in turn influenced by the weather, olive cultivar region, extraction method, and growing conditions. Previous studies have revealed that the microbiome of OP is similar to that of other olive oil by-products like olive mill wastewater (OMWW) and is composed primarily of bacteria and yeast. Proteobacteria were found to be the most prevalent microorganism, followed by *Actinobacteria (Streptomyces), Firmicutes (Staphylococcus)*, and *Acidobacteria,* according to Vivas et al. [31]. Furthermore, members of *Pseudoxanthomonas*, *Hydrocarboniphaga* and *Stenotrophomonas (Gammaproteobacteria)* were detected, with *Comamonas (Betaproteobacteria)* as the main microbial group. The cultivar seems to have a significant influence on the fungus population. The dominant yeasts were *Pichia caribbica* (syn. *Meyerozyma caribbica*), *Pichia holstii* (syn. *Nakazawaea holstii*), and *Zygosaccharomyces fermented* (syn. *Lachancea fermenta*), which were followed, to a lesser extent, by *Zygosaccharomyces florentinus* (syn. *Zygotorulaspora florentina*), *Lachancea thermotolerans* (syn. *Kluyveromyces thermotolerans*), *Saccharomyces cerevisiae*, and *Saccharomyces rosinii* (syn. *Kazachstania rosinii)* [31-33].

1.2.3 Sustainable alternatives

Currently, OP is mainly used to recover the residual oil by solvent extraction [34]. It is possible to recover the stone fragments that can be used as fuel for heating the kilns or to produce activated carbon [35,36]. Nevertheless, the OP derived from the two-phase decanter and the pitted one are difficult to manage for the oil extraction because more time and energy are necessary for pomace dehydration [37]. Thus, researchers have been focusing their findings on sustainable uses of olive pomace involving the extraction of molecules of interest, such as hydroxytyrosol, tyrosol, oleuropein, caffeic acid, and squalene, intended for cosmetic purposes, regarding their UV filter profile [38]. The olive leaf and its extracts are known for their beneficial properties for human health due to their richness in phenolic compounds [39]. These

compounds have relevant antioxidant, anticancer, antimicrobial, antifungal, and anti-inflammatory activities [40-45].

The direct use of OP has been mainly proposed for non-edible purposes, such as clay bricks, since wet OP forms pores, allowing the production of construction materials with insulation properties [46]. Due to its adsorption characteristics, OP has also been used as pollutant remover from soil, being effective against pollutants such as heavy metals and triazinic herbicides. Due to its chemical properties, composted OP has also been used as a conditioner and fertilizer [47]. Composting of solid wastes requires adjustments of conditions such as temperature, pH, moisture, oxygen level, and nutrients to permit microbial development [31,48]. A carbon-nitrogen ratio between 20 and 40 in the composting material, a moisture content of 50% to 65%, and an oxygen supply are optimal conditions for the composting process: however, they are not enough if the mass transfer during the process is limited. The main issue with this procedure using olive oil by-products is odor emission, as well as the produced wastewater, which needs to be treated. Biofilters are used to treat the emitted gas from the composting process in an effort to minimize this issue, raising the technology's overall cost [48]. This method could be a low-cost alternative to combustion for recycling solid wastes with complete decontamination of raw materials [11]. Additionally, OP is a natural source of phenolic compounds (**Table 2**) and several studies are focused on the development of new extraction methods to improve the extraction yield [49-51].

1.3 Fermentation

Fermentation is one of the most commonly used processes in the food industry; it keeps the essential qualities of the product, minimizes loss of nutrients with bioactive characteristics, and lengthens the food's shelf life [52,53]. Antimicrobial, antioxidant, and anti-inflammatory bioactivities are produced during the fermentation of plant by-products, which are important for human health [54]. The anaerobic digestion of OP biomass leads to biogas production, recovering energy, and increasing environmental sustainability [55,56]. Additionally, olive pomace has been identified as a potential substrate to produce bioethanol. Fermented olive pomace (FOP) is used as a main source of nutrients for enzyme production using solid-state fermentation by *Aspergillus* species [57,58]. Due to its nutritional value, FOP is also used as a feed supplement for animal production [59].

But knowing that this residue comes from the food industry, it would be ideal to use it for this purpose. Nowadays, studies verify that OP can be a food fortifier in bread, pasta, and granola [60,61]. The results showed that the enrichment of bread and pasta with OP improved phenolic contents and antioxidant activity before and

after the cooking process [60-62]. Research showed that the addition of polyphenol-rich extracts to dairy products increased their stability and prevented rancidity [62]. Studies also confirm the potential as a functional ingredient with prebiotic activity of OP-added formulations subjected to simulated gastrointestinal digestion followed by in vitro fecal fermentation [21,22,63]. Nunes *et al.* [22] verified that olive pomace extract can be considered an all-in-one and advantageous ingredient since it presents a mixture of lipidic and hydrophilic bioactive compounds usually not present in other plant extracts. They also verified antibacterial activity in OP against Gram-positive and Gram-negative pathogenic bacteria.

1.4 Dietary fiber and prebiotic activity relevance

Plant-based diets, such as Mediterranean or Nordic, due to their high content of dietary fiber (DF) rich foods, bear unique bioactive component blend, including antioxidants, minerals, vitamins, phytochemicals, and resistant starch. Epidemiological and clinical studies indicate that a high daily intake of vegetables and fruits is related to illness risk reduction, such as heart failure, cardiovascular and cerebrovascular events, rheumatoid arthritis, diabetes, and cancer, illnesses associated with inflammatory and oxidative body responses [64-67]. The DF and phenolic compounds present in plants provide chemopreventive activity. DF acts by reducing the exposure time of carcinogens on the gastrointestinal (GI) tract by improving the bowl movement since the majority of the DF is not assimilated by human organisms, feeding the gut microbiota. Additionally, DF delays the movement of food from the stomach to the intestines, causing a slow rise in blood glucose levels [68]. The suppression of postprandial glycemia rise avoids excessive insulin secretion and prevents fat accumulation [69]. Hence, high DF intake is related to insulin resistance and fat accumulation reduction [70,71]. The increased DF intake is also associated with anti-inflammatory and anti-cancer effects due to its prebiotic effect [72].

According to Roberfroid *et al.* [73], the prebiotic effect is defined as “the selective stimulation of growth and/or activity(ies) of one or a limited number of microbial genus (era)/species in the gut microbiota that confers health benefits to the host”. To consider that a substance has a prebiotic effect, it needs to fulfill three main requisites: not to be digested by endogenous enzymes in the human gut; to be selectively fermented by specific genera/species of gut microbiota; and to increase specific bacteria that confer benefits regarding human health [73]. The presence of prebiotic fermentation in the small intestine by probiotics strongly inhibits non intestinal prokaryotic and eukaryotic pathogens, due the production of bacteriocins,

nisins, and metabolites such as ethanol and lactic acids, hampering pathogen growth by decreasing pH [74].

Insoluble DF is composed mainly of cellulose, lignin, and other phenolic compounds and contributes to fecal bulk increase and bowel movement [75]. Colonic microbiota metabolizes soluble DF (resistant starch, pectin, and some hemicelluloses), fermenting them into short-chain fatty acids (SCFAs) (acetate, propionate, and butyrate). Recent studies demonstrate that SCFAs improve gut health by ranging from the maintenance of intestinal barrier integrity to the production of mucus and protection against inflammation, reducing the risk of colorectal cancer [76-78]. SCFAs regulate systemic functions through the inhibition of histone deacetylase activity; this intracellular signaling mechanism has a role in both gut and immune tissue responses, as well as the peripheral nervous system and the central nervous system [79,80]. Therefore, only a fraction of SCFAs from the colon reach the circulation and other tissues, and their effect on systems has been widely highlighted.

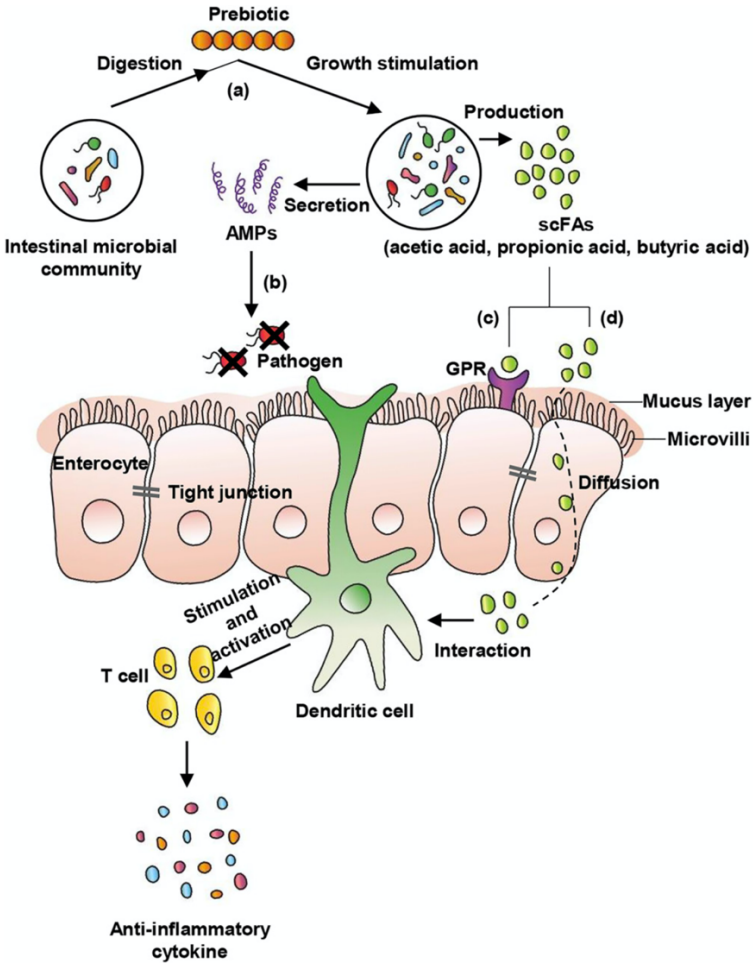

Figure 2: Activity and immunomodulatory mechanism in fish through supplementation with probiotics. (a) Binding with pathogen-active sites to reduce pathogenicity. (b) Preventing pathogen adhesion to the intestinal wall. (c) Direct interactions with dendritic cells to activate and stimulate immune cells. [81]

The most documented effect is regarding T regulatory cell differentiation and inflammatory control [82-85]. Additionally, SCFAs influence brown adipose tissue activation, regulation of liver mitochondrial function, whole-body energy homeostasis, and control of appetite and sleep [86-89]. According to Silva *et al.* [90] the SCFAs produced by the gut microbiota increase neurogenesis, improve cognitive development, improve memory, have an anti-aging effect on brain cells, improve microglia development and function, induce homeostatic profile of the microglia and reduce inflammatory signaling of microglia as well as astrocytes, thereby, improving the integrity of the blood-brain barrier and reducing its permeability - **Figure 2**.

1.4.1 OP prebiotic potential

Few studies aimed to verify the prebiotic effect of olive by-products; they evidenced a prebiotic effect on OP derivates (OP powder and bread enriched with polyphenol rich fiber (PRF) from defatted olive pomace by-product), according to Ribeiro *et al.* [63] OP powder promoted the production of SCFAs to a higher degree than fructooligosaccharides, and Nissen *et al.* [91] highlighted the health-positive effects on the host gut model: increased abundance of beneficial bacteria groups (such as Bifidobacteriaceae and Lactobacillales, increase of SCFAs production, medium-chain fatty acids (MCFAs), and reduction of excess harmful branched-chain fatty acids (BCFAs), indole, and skatole.

1.5 Objectives

The objective of this study was to evaluate the fermented olive pomace (FOP) in monocultures of *L. fermentum*, *L. paracasei,* and *L. plantarum*, to analyze if it has prebiotic effect at different fermentation timepoints, to compare them with negative and positive controls, as well as to compare short fermentation with long fermentation, and the effect of gastrointestinal digestion *in-vitro* in FOP properties.

2. Materials and Methods

2.1 Sampling

The olive pomaces were collected in two-phase olive mills in Portugal during the season 2017/2018. One olive pomace from Alfândega da Fé was selected. Olive pomaces were constituted by a mixture of olive cultivars.

2.2 Fermentation Process

The total fermentation of the OP is thirty-two days long under batch conditions, and it was evaluated by sample collection at forty-eight hours of fermentation, ninety-six hours, eight days, sixteen days, and thirty-two days. The sample were prepared using a sterile glass of 2 liters of volume, adding the OP, and closing firmly the lid. The fermentation process was done wildly — using their own microbiota — at room temperature. It is important to highlight that in this research, the OP was not diluted in water or a saline solution.

2.3 Sample preparation

2.3.1 Fermentation process

The total fermentation of the OP is 32 days long under batch conditions, and it was evaluated by sample collection at 48 hours of fermentation, ninety-six hours, eight days, sixteen days, and thirty-two days. The sample were prepared using a sterile glass of 2 liters of volume, adding the OP, and closing firmly the lid in order to, within time, generate an anaerobic environment. The fermentation process was done wildly — using their own microbiota — at room temperature. It is important to highlight that in this research, the OP was not diluted in water or a saline solution.

The selected fermentation timepoints were chosen according to the growth curve obtained earlier in the project. There were four timepoints selected: the initial one without fermentation (T0); the two-days fermentation (T2); the four-days fermentation (T4) due to the curve growth peak; and finally, the thirty-two-days fermentation (T32).

2.3.2 Controls

Three controls were done: the first control was made using only 9 mL of MRS broth without glucose (Liofilchem, Italy) and 1 mL of 0.3 McFarland monoculture suspension (negative control), and two positive controls using glucose in the second and inulin – reference prebiotic - in the third control, both at 2% concentration w/v. The controls were made by adding 0.2 g of glucose to 9 mL of MRS broth, followed by the sterilization of the solution by a cellulose filter (0.22 μm of pore size). The same steps were followed regarding inulin. The initial pH was measured using a pHmeter (Mettler Toledo) in a sterile environment to avoid contamination.

2.3.3 Simulated gastrointestinal digestion in-vitro (SGDI)

In order to be able to evaluate the actual prebiotic effect of OP, a simulated gastrointestinal digestion *invitro* (SGDI) was performed [92]. This part of the step was performed by a laboratory that is a partner in this study, as they developed and are used to techniques and the needed equipment.

Oral phase

To simulate the mastication of solid food, a manual mincer is used. A paste-like consistency is produced by adding an electrolyte stock solution with simulated salivary fluid (SSF) to the minced food. The final ratio of food to SSF needs to be 50:50 (w/v). Human salivary α-amylase is added, and the final concentration is 75 U/mL, followed by $CaCl_2$, which needs to be at 0.75 U/mL in the final mixture, and the necessary quantity of water in order to dilute the SSF solution. The contact time with the enzyme is 2 minutes at 37°C and pH 7, therefore the pre-warming of the reagents until this temperature is necessary.

Gastric phase

Five parts of the oral bolus is mixed with four parts of simulated gastric fluid (SGF) of 50:50 (v/v) after the addition of other recipients and water. Porcine pepsin is added to achieve 2000 U/mL as well as gastric lipase at 60 U/mL in the final digestion mixture, followed by $CaCl_2$ to achieve 0.075 mM in the final digestion mixture. To reduce the pH to 3, HCl 1M is added. Finally, the necessary amount of water is added to the mixture to dilute the stock solution of SGF. The digestion time is 2 hours at 37°C, with mixing. The pH may need to be readjusted with 1M HCl during digestion.

Intestinal phase

After the addition of additional receivers and water, the ultimate ratio of gastric chyme to simulated intestinal fluid (SIF) is 50:50 (v/v) by combining 5 parts of chyme with 4 parts of electrolyte stock solution from the SIF. In order to neutralize the gastric mixture to pH 7.0, the addition of 1M NaOH is needed. Bile salts (10mM) are added, followed by trypsin in pancreatin (100 U/mL). The solution incubation occurs for 2 hours at 37℃ and pH 7, while mixing.

2.3.4 Sterilization

Each timepoint of the fermentation before and after SGDI was submitted to moist heat sterilization, using autoclave.

2.3.5 Preparation of timepoints concentration

For each timepoint selected, concentrations of 2.5%, 5%, 10% and 40% w/v were prepared. In a sterile environment, by flame, the FOP was weighted using an analytical balance (0.25 g (2.5%), 0.50 g (5%), 1.00 g (10%) and 4.00 g (40%)) and added to 9 mL of MRS broth.

2.4 Prebiotic activity assay

2.4.1 Probiotic

The prebiotic assay was performed using MRS broth and a monoculture of standard probiotics: *Lactobacillus fermentum* ATCC ® 9338, *Lactobacillus paracasei subsp. paracasei* ATCC ® BAA-52, and *Lactobacillus plantarum* subsp. plantarum NCTC ® 13644.

2.4.2 Cell suspension

The cell suspension was made using a McFarland cytometer (DEN-1) to measure the optical density (OD). Fresh CFU of the selected microorganism monoculture were carefully added to a saline solution until 0.3 McFarland. The cell suspension was added (1 mL) to each control, and to each weighted sample with MRS broth. The controls and each timepoint sample were submitted to an incubation of 48 hours at 37℃. The initial pH was measured, using a pHmeter (Mettler Toledo).

2.4.3 Assay evaluation

In order to evaluate the growth of each microorganism in the controls and samples and be able to compare the results, a serial dilution (10^0 – 10^{-7}) using saline solution was performed, followed by the pour plate method of 100 μL in MRS agar (Scharlau, Spain) done in triplicate. The seeded Petri dishes were incubated for 48 hours at 37°C, and then dishes with a CFU quantity between 30 and 300 were selected to be counted. The CFU per milliliter was calculated using Equation 1, where 10^x is the effectuated dilution and 0.1 is the volume added to the media. The final pH was measured, using a pHmeter (Mettler Toledo) in order to determine if there was any acid fermentation by decreasing pH.

$$\frac{CFU}{mL} = \frac{quantity\ of\ CFU}{10^x \times 0.1} \qquad \textbf{Equation 1}$$

2.5 Statistical Analysis

One-way analysis of variance (ANOVA) was the statistical method employed to compare the results. The same assay, carried out with the same monoculture, yielded triplicate findings for the negative control and the selected sample. These results were submitted to data analysis using Excel, and statistical data was gathered.

3. Results and Discussion

3.1 Prebiotic activity assay

3.1.1 Before SGDI submission

The prebiotic activity assay with samples before SGDI submission results were different for each probiotic used. The assay performed with *Lactobacillus fermentum* showed a better development of the microorganism with an increase in OP concentration, regardless of fermentation time (**Figure 3**). There was a greater CFU per volume in the OP's inoculum, verifying the nutritional richness of this substance regarding *L. fermentum*. The relationship between microorganism growth and OP fermentation time is inverse; there is a decrease in CFU/mL of *L. fermentum* in inoculum with longer fermentation time. This result may be due to the utilization of the substrates that this bacterium uses to ferment since the fermentation process needs carbohydrates in order to happen. Therefore, the longer the fermentation, the higher the depletion in substrate availability over time.

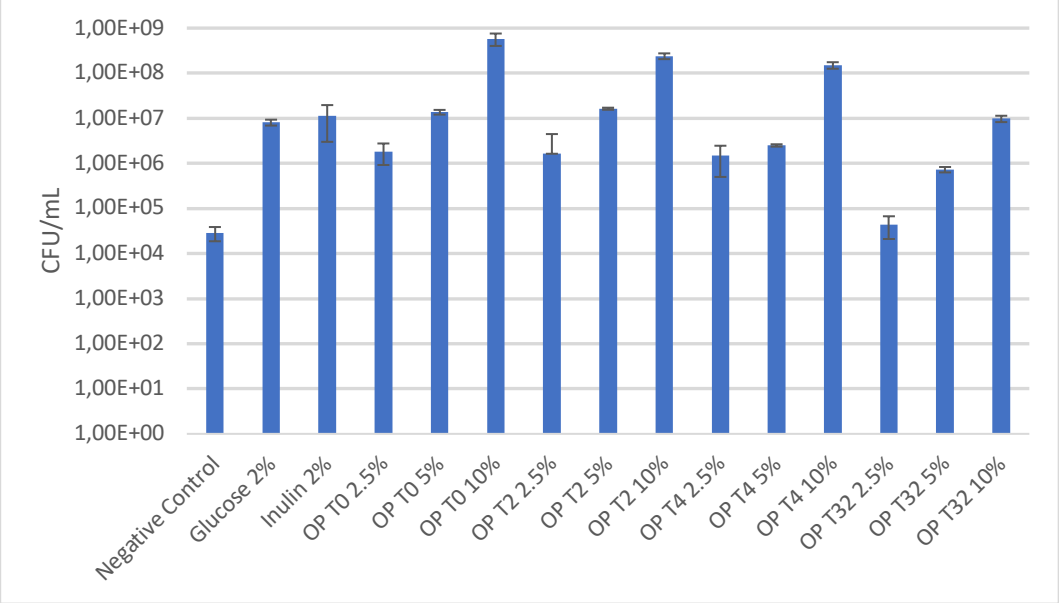

Figure 3: Prebiotic assay results using *L. fermentum*. OP: Olive Pomace; T0: without fermentation; T2: two-days fermentatiton; T4: four-days fermentation; T32: thirty-two-days fermentation; 2.5%, 5% and 10% are values relative to the concentration of each OP timepoint.

The assay performed with *Lactobacillus paracasei* showed a better development of the microorganism with a decrease in OP concentration depending on fermentation time - **Figure 4**. Although *Lactobacillus paracasei* developed better in the OP inoculum when compared to the negative control, it was verified that there

was a greater CFU per volume in the inoculums with 5% OP concentration to T0 than 10% and in the inoculum with 2.5% OP concentration to T2 and T4 than the ones with 5% and 10% OP. One hypothesis to justify this result is that it may be due to the presence of a substance or substances that inhibit this microorganism from developing, so the higher the concentration of the OP, the greater the concentration of this substance. Therefore, the evaluation of chemical composition over time is important.

Olives are rich in phenolic compounds; oleuropein, elenolic acid, verbascoside, and demethyloleuropein are the most prevalent ones. Previous studies verified the inhibition effect that oleuropein has on pathogenic bacteria when the concentration is higher than 200 μg/mL and verified that reducing the substrate (yeast extract) quantity in their test medium resulted in an inhibition of *L. plantarum, Pediococcus cerevisiae, Lactobacillus brevis,* and *Leuconostoc mesenteries* growth by oleuropein [93,94]. The abundance of substrates, such as fibers, carbohydrates, and fatty acids, in the OP T0 5% sample may overcome the concentration of inhibitor, which does not occur in the OP T0 10% sample. Elenolic acid and the aglycone of oleuropein were inhibitory to lactic acid bacteria [93,94]. The relationship between microorganism growth and OP fermentation time is inverse; there is a decrease in CFU/mL of *L. paracasei* in inoculum with longer fermentation time. This result may be due to the utilization of the substrates that this bacterium uses to ferment since the fermentation process needs carbohydrates in order to happen. Therefore, the longer the fermentation, the higher the depletion in substrate availability over time.

Phenolic compounds are known for their antioxidant activity, prebiotic effect, anticancer activity, prevention of cardiovascular and neurodegenerative diseases, and many other benefits. Therefore, it may seem incoherent to discuss the negative influence that these compounds may have on beneficial bacteria. However, the positive or negative effects depend not only on the molecule but also on its concentration. Fermentation is already an approach that proposes to change the chemical profile of the OP, mainly in order to make it more palatable to human since, due to its phenolic content, the OP has a bitter taste, while simultaneously improving the microorganism profile to make it a probiotic food.

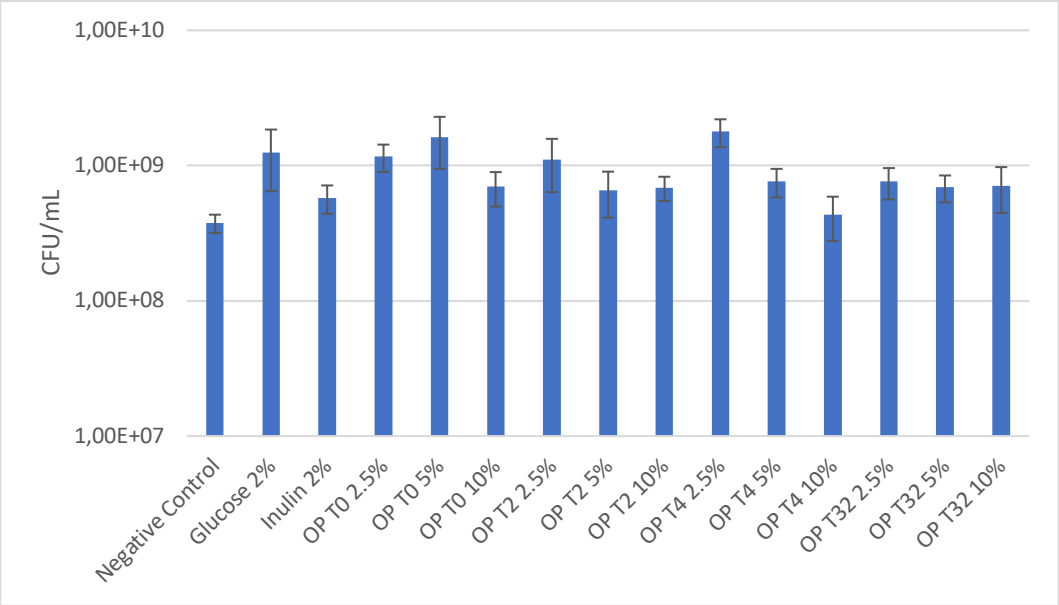

Figure 4: Prebiotic assay results using *L. paracasei*. OP: Olive Pomace; T0: without fermentation; T2: two-days fermentation; T4: four-days fermentation; T32: thirty-two-days fermentation; 2.5%, 5% and 10% are values relative to the concentration of each OP timepoint.

The results of *L. plantarum* prebiotic activity assay shows a similar relationship between microorganism development and OP concentration - **Figure 5**. With the concentration increase, a depletion of CFU per unit of volume can be assessed. There is no significant growth regarding OP T32, i.e., the CFU per unit of volume. Due the process of wild fermentation, there is a decrease in substrate content and the creation of metabolites, such as acids, alcohols, and gases that change the environment, providing different conditions over time that may decrease the content of phenolic compounds.

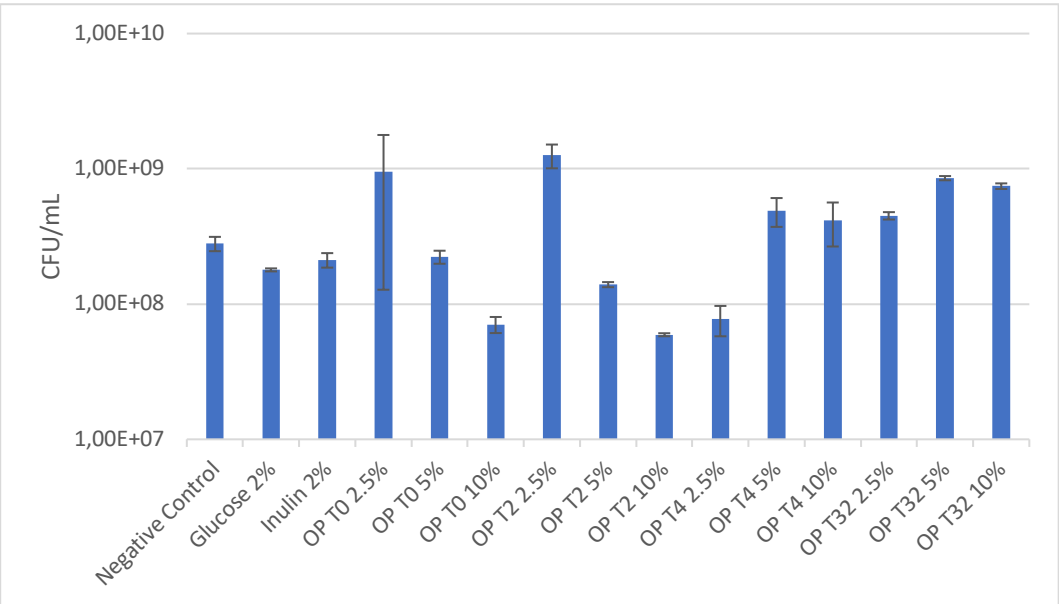

Figure 5: Prebiotic assay results using *L. plantarum*. OP: Olive Pomace; T0: without fermentation; T2: two-days fermentation; T4: four-days fermentation; T32: thirty-two-days fermentation; 2.5%, 5% and 10% are values relative to the concentration of each OP timepoint.

3.1.2 After SGDI submission

In order to confirm the prebiotic activity, the test component must reach the intestine after being exposed to the conditions that the gastrointestinal tract provides, such as the stomach acidic, digestive enzymes, and bile acid. The prebiotic activity assay was performed after submitting each OP timepoint sample to SGDI. The *Lactobacillus fermentum* results showed a greater CFU per milliliter of the microorganism in the sample with 5% OP than in the 2.5% and 10% samples, as illustrated in **Figure 6**.

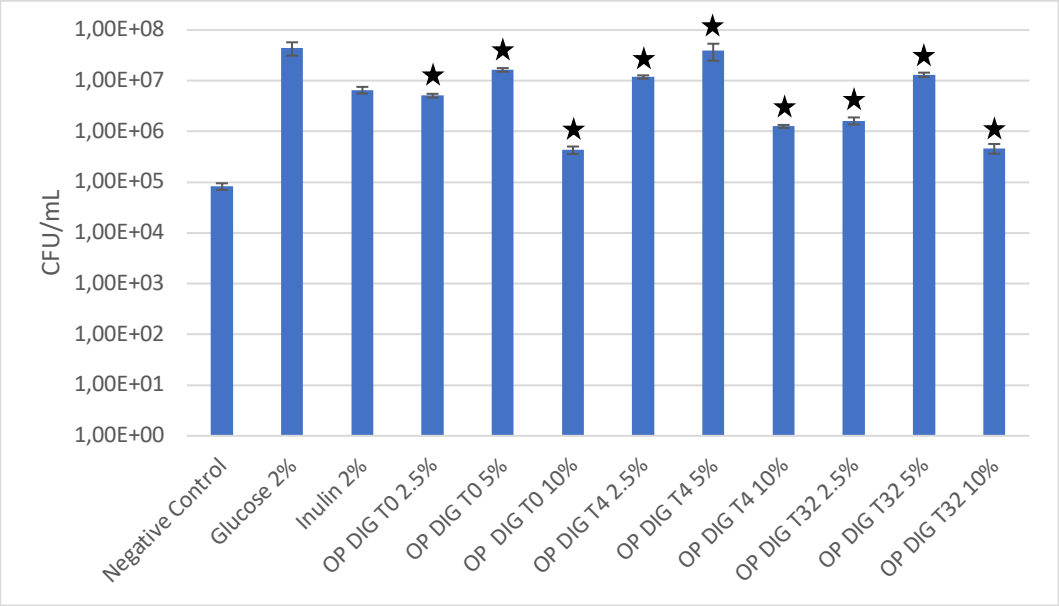

Figure 6: Prebiotic activity assay results using *L. fermentum*. OP: Olive Pomace; DIG: submitted to simulated gastrointestinal digestion *in-vitro*; T0: without fermentation; T4: four-days fermentation; T32: thirty-two-days fermentation; 2.5%, 5% and 10% are values relative to the concentration of each OP timepoint. ★ : p-value<0.001; ▲: p-value<0.05

The remaining substances, after the SGDI, are the ones that the human body cannot break down and absorb, components such as DF and phenolic compounds, hence their prebiotic effect [70,91,95-98]. The decrease in microbial growth could be due a higher concentration of phenolic compounds, overcoming the abundance of DF in the sample. Higher CFU per milliliter in the OP DIG T4 inoculum can be verified. The statistical analysis using one-way (ANOVA) to compare the negative control result (average = 8.30E4 CFU/mL) with the OP DIG T0 10% (average = 4.33E5 CFU/mL) - which was the sample with less CFU per milliliter – (p<0.05), therefore, a significant growth, verifying a prebiotic effect. The SGDI of OP in effect bacterial development can be assessed in **Figure 7**.

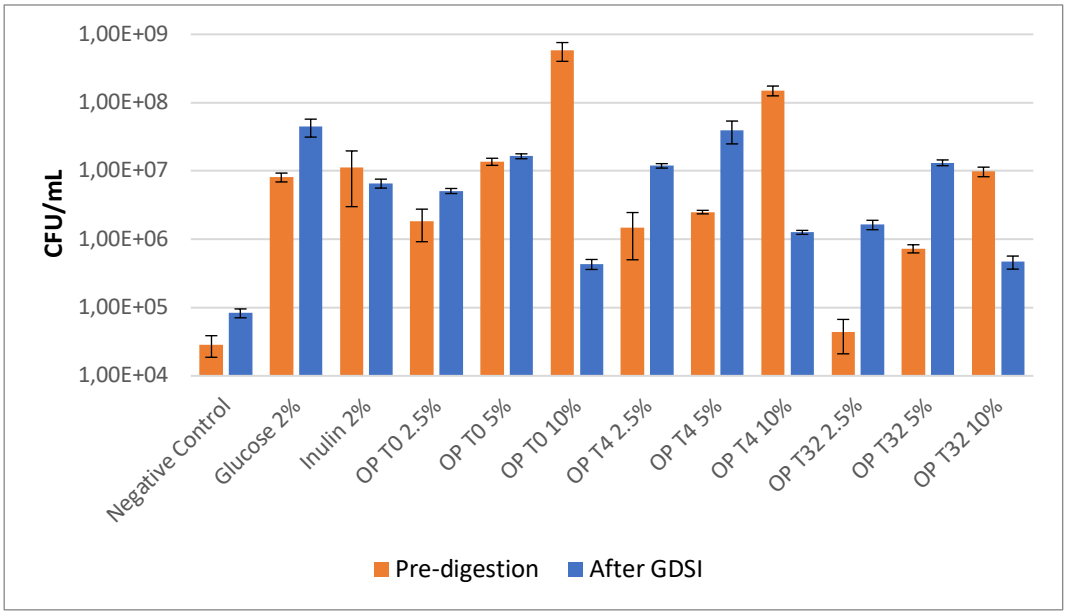

Figure 7: Comparison of prebiotic assay results before and after SGDI - *L. fermentum*. OP: Olive Pomace; DIG: submitted to simulated gastrointestinal digestion *in-vitro*; T0: without fermentation; T4: four-days fermentation; T32: thirty-two-days fermentation; 2.5%, 5% and 10% are values relative to the concentration of each OP timepoint.

The assay performed with *Lactobacillus paracasei* to evaluate the OP samples submitted to GDSI confirms the inhibition profile assessed in the prebiotic assay performed before the submission (**Figure 8**). The inoculums from OP DIG T0, OP DIG T4 and the sample OP DIG T32 10% had less growth than the negative control; the antibacterial properties of phenolic compounds may have caused an inhibition that outweighed the availability of substrate. The OP DIG T32 2.5% sample had higher growth, followed by the 5% concentration.

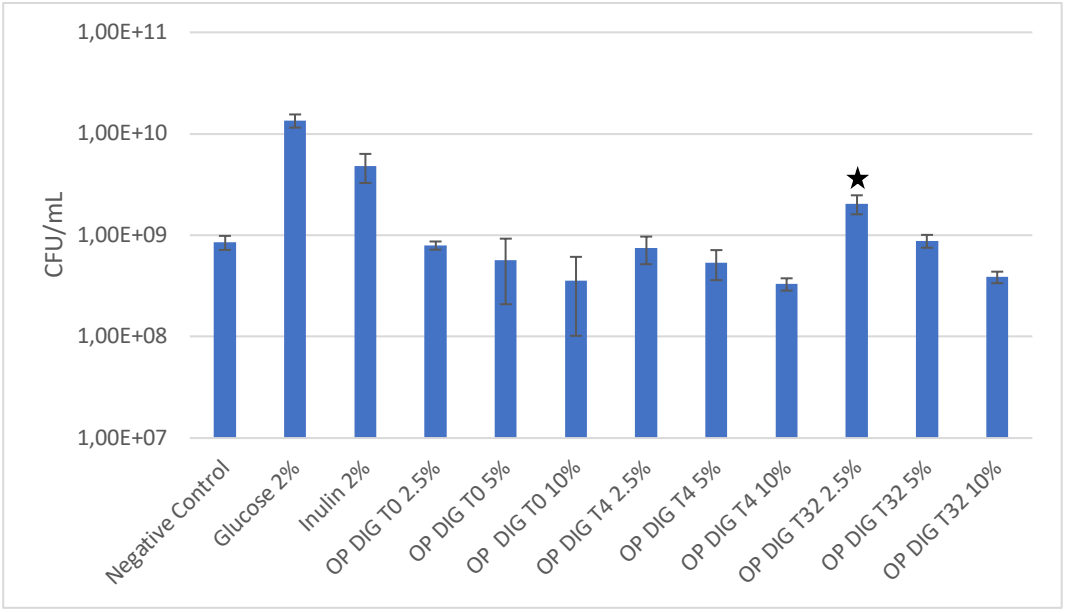

Figure 8: Prebiotic activity assay results using *L. paracasei*. OP: Olive Pomace; DIG: submitted to simulated gastrointestinal digestion *in-vitro*; T0: without fermentation; T4: four-days fermentation; T32: thirty-two-days fermentation; 2.5%, 5% and 10% are values relative to the concentration of each OP timepoint. ★ : p-value<0.001; ▲: p-value<0.05

The number of CFU per milliliter in OP DIG T32 5% had statistical irrelevancy compared to the negative control ($p > 0.05$), therefore, there was no prebiotic activity confirmed regarding the assay with *L. paracasei*. However, OP DIG T32 2.5% was statistically different from the negative control ($p < 0.05$). Therefore, regarding *L. paracasei*, the only sample that had a prebiotic effect was the OP at 2.5% concentration that was fermented for 32 days. The SGDI of OP in effect bacterial development can be assessed in **Figure 9**.

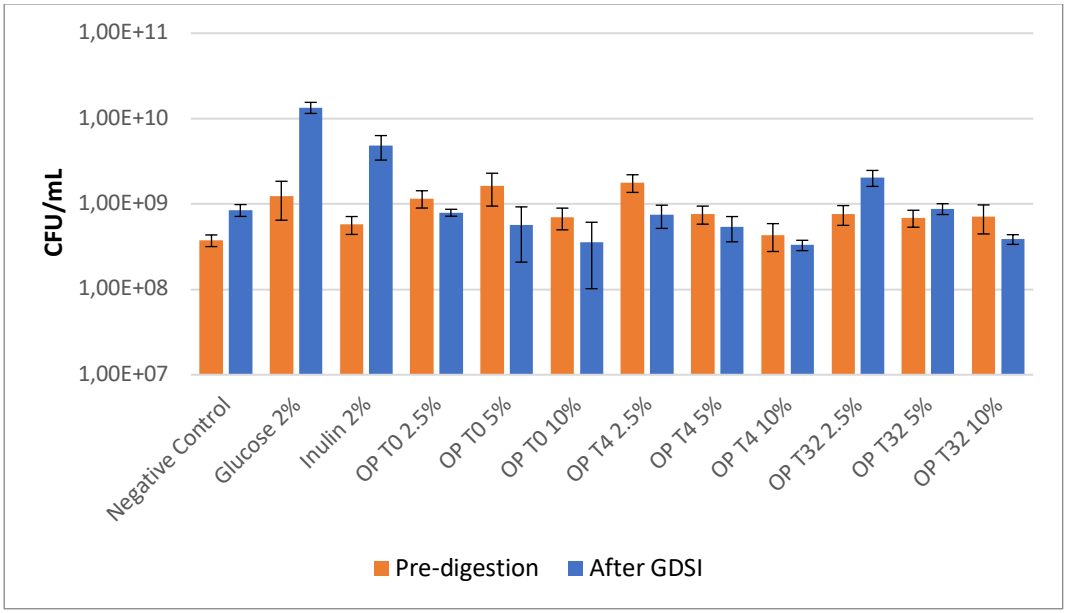

Figure 9: Comparison of prebiotic assay results before and after SGDI - *L. paracasei*. OP: Olive Pomace; DIG: submitted to simulated gastrointestinal digestion *in-vitro*; T0: without fermentation; T4: four-days fermentation; T32: thirty-two-days fermentation; 2.5%, 5% and 10% are values relative to the concentration of each OP timepoint.

The performed assay with *Lactobacillus plantarum* showed that prebiotic activity of OP samples submitted to GDSI had a different profile compared to the ones after the submission (**Figure 10**).

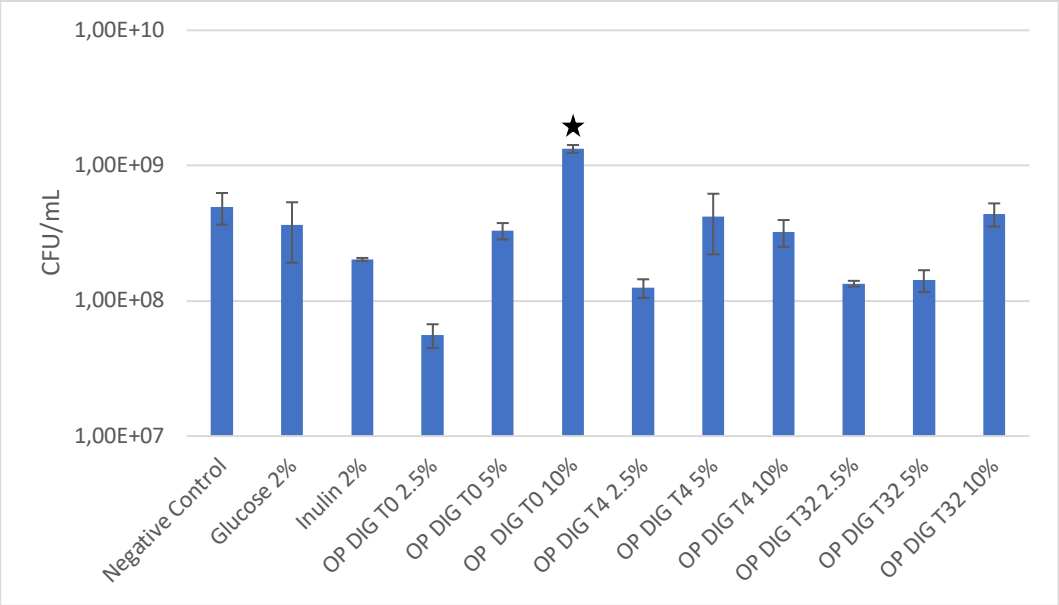

Figure 10: Prebiotic activity assay results using *L. plantarum*. OP: Olive Pomace; DIG: submitted to simulated gastrointestinal digestion *in-vitro*; T0: without fermentation; T4: four-days fermentation; T32: thirty-two-days fermentation; 2.5%, 5% and 10% are values relative to the concentration of each OP timepoint. ★: p-value<0.001; ▲: p-value<0.05

The CFU per milliliter of *L. plantarum* in OP DIG T0 inoculums increased along with the concentration. The OP DIG T4 had higher growth in the 5% sample, followed by 10% and then 2.5%. The only sample that had a CFU per milliliter higher than the negative control (average = 4.97×10^8 CFU/mL) was the OP DIG T0 10% (average = 1.33×10^9 CFU/mL): the difference was statistically relevant ($p < 0.05$). Hence, for *L. plantarum*, there is a prebiotic effect of non-fermented OP at 10% concentration. The SGDI of OP in effect *L. plantarum's* development can be assessed in **Figure 11**.

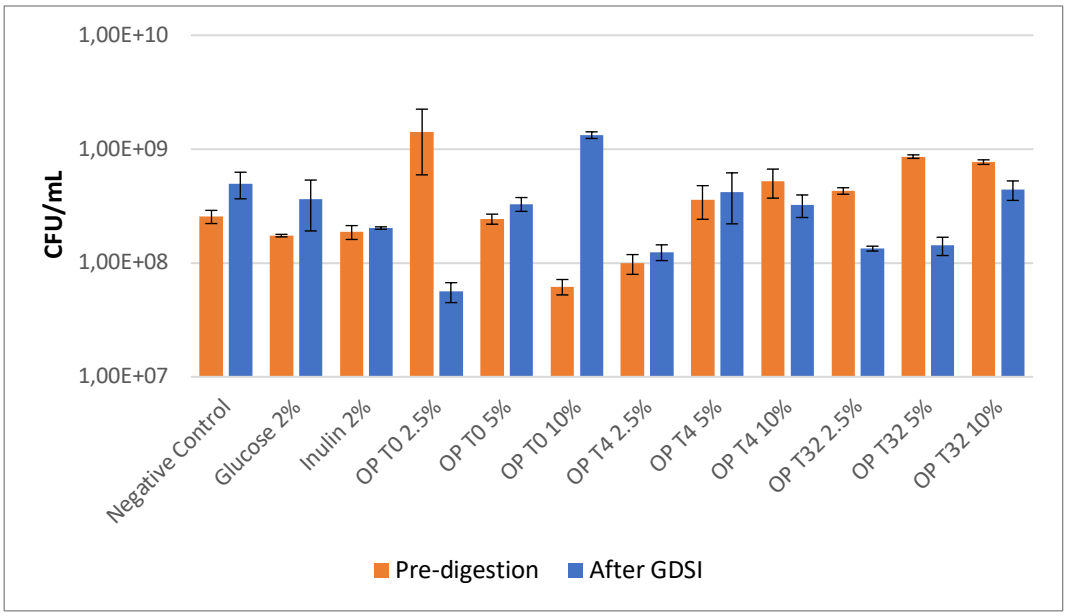

Figure 11: Comparison of prebiotic assay results before and after SGDI - *L. plantarum*. OP: Olive Pomace; DIG: submitted to simulated gastrointestinal digestion *in-vitro*; T0: without fermentation; T4: four-days fermentation; T32: thirty-two-days fermentation; 2.5%, 5% and 10% are values relative to the concentration of each OP timepoint.

After the GDSI, each sample was liquid and had a different aspect from the pre-GDSI sample, which was a paste; therefore, the distribution of DF and phenolic compounds may differ. An exploratory assay using OP DIG at 40% concentration was performed to evaluate the microorganism response. The results obtained can be assessed in **Figure 12**. The *L. fermentum* inoculums evaluated had a significant growth of almost 3 logs of difference from the negative control. A one-way ANOVA was used to compare the OP DIG 40% inoculums with the negative control. The sample without fermentation had a statistical difference ($p < 0.05$), as did the inoculums with fermentation, 4 days and 32 days. The number of CFU per milliliter was higher in OP DIG T4 40% ($1.22x10^8$ CFU/mL), followed by OP DIG T32 40% ($1.14x10^8$ CFU/mL), than in OP DIG T0 40% ($1.08x10^8$ CFU/mL). Hence, it was verified that for *L. fermentum* the fermentation process enhances the OP prebiotic activity.

The inoculums in which *Lactobacillus paracasei* was used as monoculture, the quantity of CFU per milliliter was lower than the one used in the negative control. Therefore, no prebiotic activity was verified in the assay using 40% of each sample (T0, T4 and T32) submitted to GDSI using monoculture of *L. paracasei*. The inoculums OP DIG T4 40% (average = $9.60x10^8$ CFU/mL) and OP DIG T32 40% (average = $1.34x10^9$ CFU/mL) regarding *Lactobacillus plantarum*'s assay had a quantity of CFU per milliliter significantly higher than the negative control, and the quantity of OP DIG T0 40% (average = $6.17x10^8$ CFU/mL) was also higher but not statistically different.

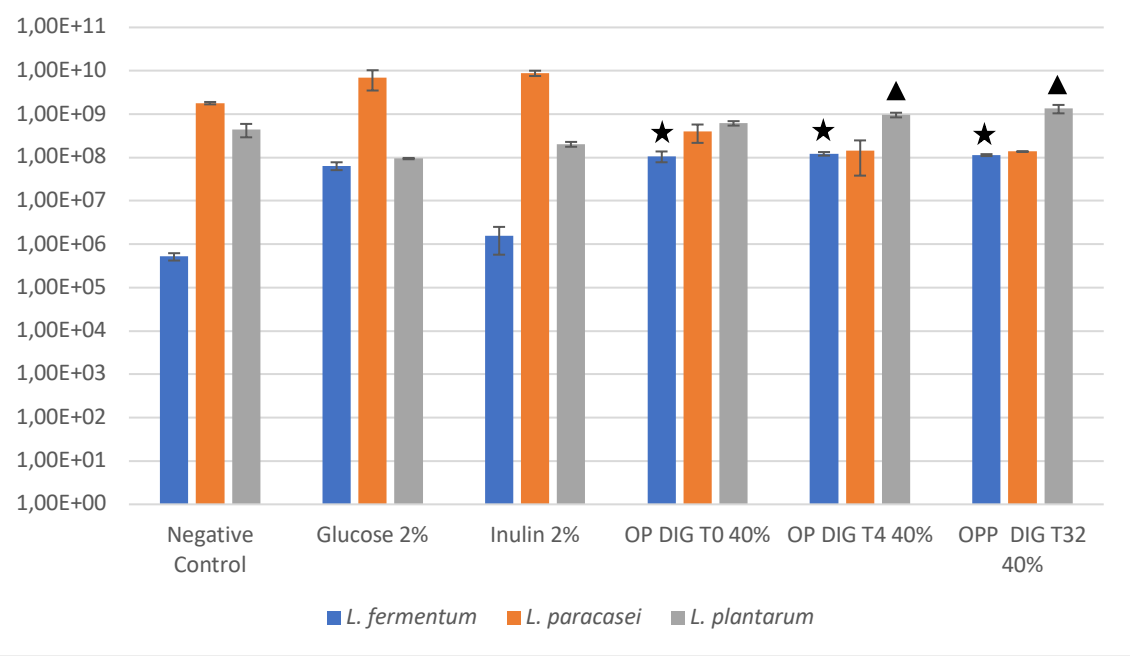

Figure 12: Prebiotic activity evaluation with 40% of OP - *L. fermentum*, *L. paracasei* and *L. plantarum*. OP: Olive Pomace; DIG: submitted to simulated gastrointestinal digestion *in-vitro*; T0: without fermentation; T2: two-days fermentation; T4: four-days fermentation; T32: thirty-two-days fermentation; 40% are values relative to the concentration of each OP timepoint. ★: p-value<0.001; ▲: p-value<0.05

Therefore, the OP fermentation time increased the prebiotic effect in *L. plantarum's* assay. Through these exploratory assays, the inhibitory action of the OP on *L. paracasei* could be assessed. Additionally, the OP prebiotic effect *in L. fermentum* was verified, as well as in *L. plantarum*, depending on the fermentation timing and concentration conditions. The quantity of CFU/mL in the inulin and glucose controls by the pour plate method showed similar counting; however, there was a greater pellet in the glucose control when compared to the inulin and comparing to the remaining inoculums in every assay. A higher pH depletion in the glucose control was detected as well -**Table 3**.

Table 3: Measurement of the initial and final pH after the incubation with each monoculture and the difference.

	L. fermentum			*L. paracasei*			*L. plantarum*		
	initial	*final*	*difference*	*initial*	*final*	*difference*	*initial*	*final*	*difference*
	pH	pH	pH	pH	pH	pH	pH	pH	pH
Negative control	6.32	6.23	-0.09	6.20	5.79	0.41	6.20	5.58	0.62
Glucose 2%	6.14	3.80	-2.34	6.14	3.10	3.04	6.14	3.14	3.00
Inulin 2%	6.30	6.26	-0.04	6.21	3.06	3.15	6.21	5.59	0.62
OP DIG T0 2.5%	6.25	6.16	-0.09	6.25	5.62	0.63	6.25	5.88	0.37
OP DIG T0 5%	6.22	6.15	-0.07	6.22	5.55	0.67	6.22	5.23	0.99
OP DIG T0 10%	6.23	6.28	0.05	6.23	5.45	0.78	6.23	4.93	1.30
OP DIG T4 2.5%	6.24	6.27	0.03	6.24	5.66	0.58	6.24	5.48	0.76
OP DIG T4 5%	6.22	6.19	-0.03	6.22	5.61	0.61	6.22	5.28	0.94
OP DIG T4 10%	6.23	6.25	0.02	6.23	5.57	0.66	6.23	4.99	1.24
OP DIG T32 2.5%	6.23	6.25	0.02	6.23	5.73	0.50	6.23	5.54	0.69
OP DIG T32 5%	6.22	6.21	-0.01	6.22	5.66	0.56	6.22	5.33	0.89
OP DIG T32 10%	6.24	6.32	0.08	6.24	5.67	0.57	6.24	5.11	1.13

The similar quantity of CFU per volume may be due to cellular death by acidification of the environment; therefore, performing the same protocol using a buffer is required. According to the literature, the standard protocol used to evaluate a substance's prebiotic effect is the evaluation of the prebiotic activity score, which can be assessed by the measurement of the optical density difference - **Equation 2**. Due to the OP's physical properties, the initial solution (medium with the OP) is already turbid, so the usage of OD could not be assessed due to results inaccuracy. The protocol used in this study had an exploratory approach and was based on Slizewska *et al* article [99].

$$\text{Prebiotic activity score} = \frac{\frac{\text{probiotic log O. D. on the prebiotic at 24hrs} - \text{probiotic log O. D. on the prebiotic 0h}}{\text{probiotic log O. D. on glucose 24h} - \text{probiotic log O. D. on glucose 24h}}}{\frac{\text{enteric log O. D. on the prebiotic at 24hrs} - \text{enteric log O. D. on the prebiotic 0h}}{\text{enteric log O. D. on glucose 24h} - \text{enteric log O. D. on glucose 24h}}}$$

Equation 2

Therefore, it would be advisable to utilize citrate-phosphate buffer in future assays in order to obtain a precise count of CFU per milliliter, without interference from pH oscillation [100]. Additionally, since inulin is not hydrosoluble, medium sterilization by cellulose filters is a challenge, and it is probable that part of the inulin is retained on the membrane. The utilization of fructooligosaccharides (FOS) in the positive prebiotic control to replace inulin would be a better approach [100]. FOS are hydrosoluble, as is glucose, making them easier to filter and, consequently, less likely to be retained during the filtration process.

4. Conclusion

In this study, it was possible to verify the potential for prebiotic effects from fermented and non-fermented OP. The aim of the project is the valorization of this by-product, highlighting its nutritional and microbiological potential to nourish human beings. Through exploratory assays, the prebiotic effect of the OP before its submission to GDSI in *L. fermentum* could be verified, and later, after the submission of the sample, the profile remained coherent with the one that was previously assessed. Regardless of the concentration and fermentation time, this microorganism's growth was enhanced in the presence of this by-product. The decrease of the prebiotic effect within fermentation time was verified, as was the increase with OP concentration. The decline in CFU per milliliter during fermentation time could be attributed to the consumption of substrates by *L. fermentum* throughout the process. Consequently, as the duration of the process increases, the availability of the substrate diminishes. Nonetheless, the inhibitory effect of the OP regarding *L. paracasei* could also be assessed, it was possible to observe in the assay with the sample before and after being submitted to GDSI. The growth of this strain was inhibited according to the increase in OP concentration, but less affected within fermentation time. Due to fermentation, inhibitory components may be degraded. Additionally, the OP prebiotic effect in *L. plantarum*, depending on the fermentation time and concentration conditions, could be verified. The prebiotic effect of OP on *L. plantarum* was enhanced by fermentation time, contrary to the *L. fermentum* results. This may occur due to many factors: degradation of inhibitory chemicals, availability of substrates that *L. plantarum* could not break down in its complete form but has enzymes that catabolizes oligomers or smaller particles, fermentation metabolites are produced, and *L. plantarum* may use them as energy sources, etc. In conclusion, this work allowed the verification of OP as a potential prebiotic source.

5. Future work

Nevertheless, there is still much work to be done. The improvement of the used protocol was to add buffer in each assay, as well as replace inulin for FOS, and to perform the assays with more types of probiotic bacteria, such as *Pediococcus cerevisiae*, *Leuconostoc mesenteries,* and *Bifidobacterium longum*, as well as probiotic yeast, such as *Saccharomyces boulardii*, and enteric bacteria (*Escherichia coli*), in order to calculate the prebiotic activity score of each OP sample. The analysis of inoculums with lower OP concentrations in *L. paracasei* monoculture would be interesting in order to try to verify if there is a concentration at which this by-product exerts a relevant positive effect on these bacteria. Additionally, further studies should aim to analyze the influence of OP in the human gut microbiota by performig a protocol using fresh human stool and different concentrations of OP after being submitted to simulated gastrointestinal digestion *in vitro*.

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