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VALORIZATION OF IMMATURE WASTED TOMATOES TO INNOVATIVE FERMENTED FUNCTIONAL FOODS

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Declaration of Authorship of work

I declare to be the author of this work, which is original and unpublished. Consulted authors and papers are properly cited in the text and are included in the included reference list.

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ABSTRACT

This study aimed to consolidate a strategy to valorise immature tomato fruit (GT, *cv.* H1015) through controlled fermentation (use of starter cultures) in producing high-value food products to support circular economy-oriented innovation. The probiotic character of two pure LAB strains, *Lactiplantibacillus plantarum* (LAB97, isolated from GT) and *Weissella paramesenteroides* (C1090, INIAV collection), were tested using static *in vitro* gastrointestinal digestion model (sequential digestion and digestive enzymes). Both LAB strain counts reached ca. 6 log CFU/ml after the *in vitro* simulation, meeting the viability criterion for potential probiotic capacity. In the evaluation of GT-controlled fermentation, the two starters (per se) and the addition of NaCl (1.5%) were assessed (10^8 CFU/ml of inoculum, 100 rpm, 20 °C, 14 days). It was concluded that LAB 97 strain was superior to the C1090 strain or spontaneous fermentation because it increased process efficiency (fast acidification) and developed an ingredient with sensory acceptance and probiotic potential (> 7 log CFU/ml). The second approach aimed to evaluate the formulation of a sauce with sensory, nutritional, and probiotic potential based on the combination of fermented GT (LAB 97) with other valuable ingredients (avocado, parsley, and honey). The formula chosen included fermented GT (65%) and a 4:2:1 mixture of these ingredients. Different technological strategies (thermal treatment and non-treatment) were tested to prevent microbial contamination by the additional ingredients and promote the shelf life of the sauce storage. The sauce's shelf stability samples were evaluated during storage (5 °C, 21 days) concerning several quality attributes (microbial counts, pH, soluble solids content, CIELab, total phenolic content, and antioxidant activity and panel sensory analysis). The viability of a sauce prototype with sensory quality and valuable antioxidant composition, meeting the microbiological criteria for this type of product, could be concluded. However, decontamination treatments do not improve sauce stability compared to raw ingredients.

Keywords: Lactic Acid Bacteria, Lactic Fermentation, *Lactiplantibacillus plantarum*, *Weissella paramesenteroides*, Probiotic Potential, Food Formulation

RESUMO

Este estudo visou consolidar uma estratégia de valorização para frutos imaturos de tomate-indústria (TV, *cv.* H1015) através da fermentação lática controlada (utilização de culturas de arranque - *starters*) no desenvolvimento de produtos alimentares de elevado valor, no âmbito da economia circular. O carácter probiótico de duas estirpes de bactérias ácido lácticas (BAL) puras, *Lactiplantibacillus plantarum* (BAL97, isolado do TV) e *Weissella paramesenteroides* (C1090, colecção de culturas do INIAV), foi testado utilizando um modelo de digestão gastrointestinal estático *in vitro* (digestão sequencial e enzimas digestivas). Após a simulação *in vitro*, ambas as estirpes BAL obtiveram contagens de *ca.* 6 log UFC/mL, cumprindo o critério de viabilidade para serem consideradas com potencial capacidade probiótica. Na avaliação da fermentação de TV, foram avaliadas as duas estirpes de BAL enquanto culturas *starters* (de per se) e a adição de NaCl (1,5%) (10^8 UFC/ml de inóculo, 100 rpm, 20 °C, 14 dias). Concluiu-se que a estirpe BAL 97 teve um desempenho superior em comparação com a estirpe C1090 ou, com a fermentação espontânea dos frutos porque contribuiu para uma fermentação mais eficiente (acidificação rápida) dando origem a um ingrediente com aceitação sensorial e potencial probiótico (> 7 log UFC/ml). A segunda abordagem visou avaliar a formulação de um molho com elevado valor composicional e bioativo, aceitação sensorial, e capacidade probiótica baseado na mistura de fermentados de TV (BAL 97) com outros ingredientes valiosos do ponto de vista nutricional e bioativo (abacate, salsa e mel). A formulação selecionada incluiu a mistura de fermentados de TV (65%) com os outros ingredientes mencionados (35%), na proporção relativa (4:2:1). Foram testadas diferentes estratégias tecnológicas (duas intensidades de tratamento térmico e ausência de tratamento) por forma a prevenir a contaminação microbiana pelos ingredientes adicionais e, em consequência promover a estabilidade do molho durante o armazenamento. O protocolo de avaliação das diferentes amostras durante o armazenamento (5 °C, 21 dias) incluiu vários atributos de qualidade (contages microbianas, pH, teor de sólidos solúveis, parâmetros da cor CIELab, teor fenólico total, capacidade antioxidante e análise sensorial por painel). Conclui-se da viabilidade do protótipo desenvolvido, obtendo-se um molho com qualidade sensorial aprovada e com composição antioxidante valiosa, cumprindo simultaneamente os critérios microbiológicos para este tipo de produto. Os tratamentos de descontaminação dos

ingredientes não contribuíram para incrementar a estabilidade do molho em comparação com a utilização dos mesmos em cru.

Palavras-chave: Bactérias do ácido láctico, Fermentação láctica, *Lactiplantibacillus plantarum*, *Weissella paramesenteroides*, potencial probiótico, desenvolvimento de novos produtos

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LIST OF ABBREVIATIONS

AOx	Antioxidant capacity
CFU	Colony Forming Unit
cv.	Cultivar
DPPH	2,2-diphenyl-1-picrilhydrazil
FRAP	Ferric Reducing Antioxidant Power
FW	Fresh Weight
GAE	Gallic Acid Equivalents
GI	Gastrointestinal
GT	Green Tomato
HPLC	High Performance Liquid Chromatography
LAB	Lactic Acid Bacteria
LD	Limit of Detection
LQ	Limit of Quantification
MRS	Man Rogosa & Sharpe
NaCl	Sodium Cloride
PSB	Phosphate-saline buffer
rpm	Round per minute
SGF	Simulated Gastric Fluid
SIF	Simulated Intestinal Fluid
SSC	Solid Soluble Content
SSF	Simulated Salivary Fluid
TA	Total Acidity
TAPC	Total Aerobic Plate Counts
TCD	Total Colour Difference
TEAC	Trolox Equivalents
TPC	Total phenolic compounds
WI	White Index
Y&M	Yeast and Moulds

THEORETICAL PART

1. LITERATURE REVIEW

Food waste and losses worldwide, estimated at one-third of world production (FAO, 2011), remain major global problems with negative impacts on the economy, nutrition, food security, and the environment. Food loss occurs at all stages of the food supply chain, from the agricultural phase up to the transformation and transport phases. Waste losses regarding fruit and vegetables were estimated to be around 40-50% worldwide (FAO, 2015).

Some management strategies have been developed in the last few years to reduce waste during agricultural production, food processing, and consumption. The European Community (EC) has recently adopted a Circular Economic Action Plan to address these issues, where policy measures to improve waste recovery focus on obtaining value-added products (EC, 2020).

Currently, the tomato (*Solanum lycopersicum* L.) harvest is one of the most important crops in the world. In 2018 its annual production was estimated to be around 17 million tons in Europe (especially Italy, Spain, and Portugal) and 182 million tons worldwide (FAOSTAT, 2018). In Portugal, the tomato industry focuses on obtaining a single high-value product – tomato paste. Only fruits at the red ripe stage (fully red mature) enter the processing plant. Consequently, around 112 M tons of green tomatoes are left in the fields yearly. The industry also rejects many fruits due to the high-quality standards required for processing tomato paste. This set of discarded fruits represents a unique opportunity to produce commercial value-added products. For these reasons, numerous global and national initiatives have emerged to reduce food waste along the supply chain by implementing sustainable management measures and strategies.

Fermented products have a set of appealing sensory characteristics (flavour, aroma, and texture) and are considered safe foods with prolonged storage time. These benefits are very competitive in the current food market, to which advantages can be added beyond the nutritional and bioactive component, namely its probiotic potential. The growing lactose intolerance and restrictions on cholesterol in fermented dairy products open a window of opportunity for developing non-dairy fermented products. Developing new products and food ingredients through fermentative processes of plant-based matrices has been widely investigated as a viable resource for reducing food waste. In

this regard, the valorisation of unripe tomatoes (a by-product of the tomato paste industry) to produce food ingredients and formulations in the fermented food group contributes to diversifying food systems and diets, improving human health and contributing to healthy ecosystems.

1.1. Food Waste Valorisation

The continued expansion in population, combined with technological developments, has created a demand-supply imbalance, leading to rising food waste worldwide. Food loss and food waste (FLW) negatively impact society and the economy. More importantly, it represents a threat to the environment and a severe operational problem for the production plants (Goula and Lazarides, 2015; FAO, 2019). Waste is frequently generated at numerous stages throughout the food supply chain. These stages are post-production, handling and storage, manufacturing, wholesale and retail, and consumption (Ravindran and Jaiswal, 2016). According to the Food and Agriculture Organization of the United Nations (FAO) predictions in 2011, Over a third of the food production is wasted along the food supply chain before reaching the consumer.

Agricultural wastes must be turned into food items since they provide a low-cost source of dietary fibre, protein, and bioactive substances such as phenolic compounds, antioxidants, minerals, and vitamins. (Peschel *et al*., 2006; Santana-Méridas *et al*., 2012; Ravindran and Jaiswal, 2016; Wang *et al*., 2019). Using by-products can reduce negative environmental impacts while also improving the nutritional profile of food for human consumption (Lai *et al*., 2017; Majerska *et al*., 2019).

Food waste management is a moral challenge for society. While many communities treat it as a severe issue, their adopted strategies are varied. For example, the European waste management hierarchy has announced a program that includes steps for waste prevention, reuse, material recovery and recycling, energy recovery, and safe landfill residues (Monier *et al*., 2010). The EC recently adopted a new Circular Economic Action Plan to ensure that the resources remain in the economy to the furthest extent possible (EC, 2020). These policies aim to improve waste valorisation and concentrate on getting value-added products.

1.2. Tomato

1.2.1. Production and Consumption

Currently, the tomato (*Solanum lycopersicum* L.) crop is the second most important fruit or vegetable crop next to potato (*Solanum tuberosum* L.), with approximately 182.3 million tons of tomato fruit produced on 4.85 million ha each year (FAOSTAT, 2019). Asia accounts for 61.1% of global tomato production, while Europe, America, and Africa produced 13.5%, 13.4%, and 11.8% of the total tomato yield. Tomato consumption is centred in China, India, North Africa, the Middle East, the United States, and Brazil, with per capita tomato consumption varying between 61.9 and 198.9 kg (FAOSTAT, 2019). In addition to being served as a fresh vegetable, tomato is also consumed in the form of various processed products, such as paste, juice, sauce, puree and ketchup (Kaur *et al*., 2008). (Figure 1.1)

The harvesting of the fruit is fully mechanised in manufacturing high-quality tomato paste. Harvesting machines equipped with sensors detect the colour of the tomatoes, leaving all green, yellow and yellow-orange tomatoes in the field (Gould, 1992). In Portugal, according to the Portuguese Centro de Competências para o Tomate de Indústria, in 2015, 1.12×10^8 kg of green, yellow, and orange-yellow tomatoes were abandoned in the fields. The non-use of these fruits represents economic losses for the producers and a waste of environmental resources. As a result, it will be critical to collect and analyse the losses of fruits created across the tomato value chain (Parfitt *et al*.,2010) and devise measures to improve the crop's economic viability. Functional ingredients for food items and raw materials for nutritional supplements are unique opportunities to provide commercial value-added products from discarded tomatoes. (Lu *et al*., 2019).

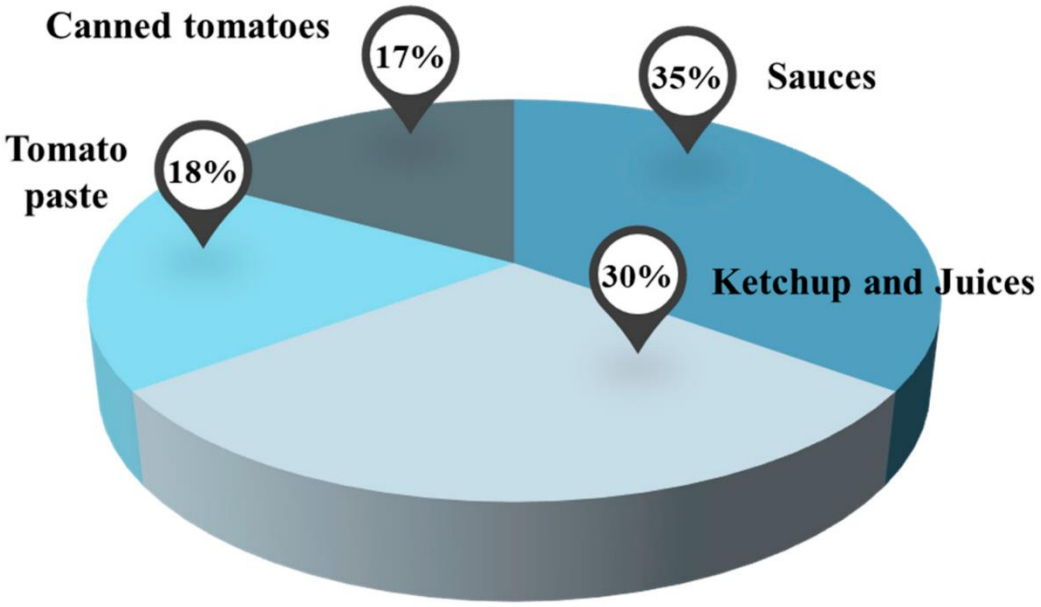

Figure 1.1 - Graphic representation of USDA's estimated percentages of tomato processed by-products. (Raiola *et al*., 2014)

Tomato is a climacteric fruit, which means it undergoes an increase in respiration and ethylene production at the onset of ripening with dramatic metabolic changes during fruit development. Several metabolic pathways are activated during the ripening process, determining the fruit's quality. These include influencing pigment levels and consequent changes in colour (e.g. lycopene accounting for the red colour), changes in the content of sugars, acids and volatiles associated with aroma, and promoting softening and tissue degradation to allow the more accessible release of the seeds.

The fruits contain 90–95% water and 5–10% dry matter, mainly sugars (50%), organic acids, and amino acids (15%) (Davies *et al*., 1981). Tomatoes' nutritional value is explained mainly by the different health-promoting components, such as vitamins, carotenoids, and phenolic compounds. (Raiola *et al*., 2014; Liu *et al*., 2016; Martí *et al*., 2016; Li *et al*., 2018). Bioactive antioxidant compounds play significant health effects in the human diet, such as anti-inflammatory, anti-allergic, antimicrobial, vasodilatory, antithrombotic and cardioprotective (Raiola *et al*., 2014). Tomatoes are rich in carotenoids, representing the primary source of lycopene in the human diet (Viuda-Martos *et al*., 2014). Carotenoids and polyphenolic compounds contribute to the nutritional value of tomatoes and improve their functional attributes and sensory qualities, including taste, aroma, and texture (Raiola *et al*., 2014; Tohge and Fernie, 2015; Martí *et al*., 2016). Tomatoes also have naturally occurring vitamins C and E (Agarwal

and Rao, 2000; Martí *et al*., 2016) and large amounts of sugars and organic acids, such as sucrose and, hexoses citrate, malate (Li *et al*., 2018).

1.2.2. Physical-chemical composition during the maturation

Tomatoes are available in six stages of maturity: mature green, breaker, turning, pink, light-red, and red (Figure 1.2). The chemical composition of tomatoes varies significantly depending on several factors: cultivar, maturity at harvest, ripening phase, and environmental conditions. However, the maturity stage at harvest and cultivar (cv) are considered the two main factors affecting the tomato's nutritional value and its quality attributes, namely the soluble solids content, titratable acidity, sugar content, colour, and firmness (Bartkiene *et al*., 2015; Moneruzzaman *et al*., 2008).

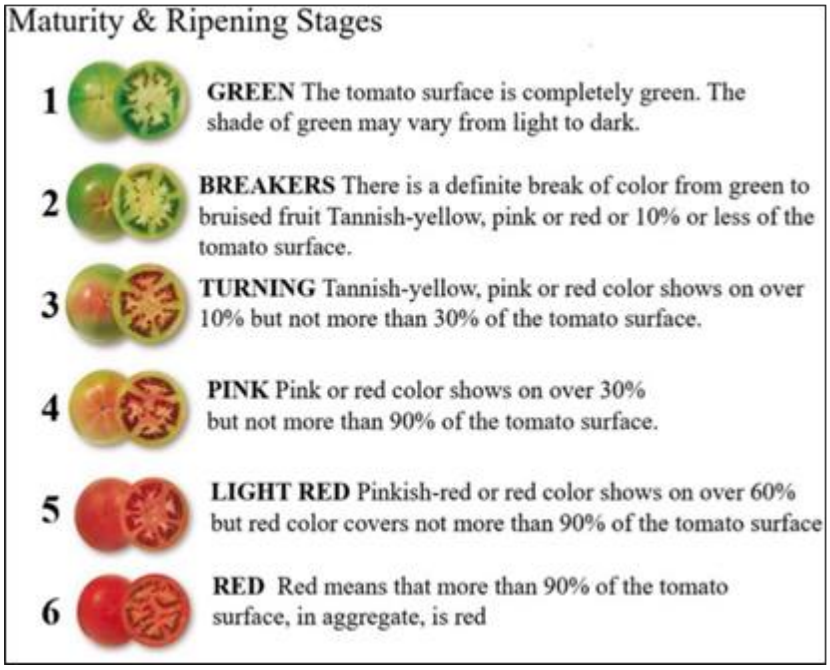

Figure 1.2 - Changes in the colour of tomato fruit during the ripening process.

Usually, total tomato acidity is low in immature-green fruits; it reaches a maximum at the turning stage and decreases rapidly afterwards (Getinet *et al*., 2008). As ripening progressed, firmness diminished either on the vine or post-harvest (Tilahun *et al*., 2017). Bui *et al*. (2010) determined the strength of deformation curves and firmness of tomatoes as a function of maturity. Accordingly, Knee and Finger (1992) reported a peak in the content of organic acids at the pink ripening stage. The sugar content of tomato fruit tends to increase from small and green mature to large and red-ripe tomatoes (Sinaga, 1986). Sammi and Masud (2007) also observed that fruits displayed a peak of sugars

during the transition from the green to the turning maturity stages. The mainly reducing sugars in tomatoes, fructose, and glucose (are generally correlated with soluble solids content - °Brix) tend to increase throughout maturation in parallel with colour (Atta-Aly *et al*., 2000; Jimenez *et al*, 1996; Znidarcic & Pozrl, 2006). Carotenes transfer the light energy they absorb to chlorophyll for photosynthesis and protect plant tissues from the damaging effects of ultraviolet irradiation. Carotenoids are natural red, orange, or yellow pigments, synthesised by various plants and microorganisms. They have long been used in cosmetics, food and feed industries for their proven biological activities such as antioxidant and pro-vitamin A. The ripening stage at harvest exerts a relevant positive effect on carotenoid levels and affects the lycopene content significantly in the red ripe stage (Helyes *et al*., 2006). Lutein, lycopene, α- and β-carotene, zeaxanthin and b-cryptoxanthin are essential carotenoids that may compose the human diet (Alda *et al*., 2009). Lycopene has the highest antioxidant activity among all dietary antioxidants (George *et al*. 2004). During the ripening process, lycopene content increased from the breaker to the red stage, while lutein displayed the reverse accumulation pattern, with higher values during the breaker stage. In contrast, beta-carotene showed the highest synthesis levels in the pink and light red stages (Bhandari *et al*., 2016).

According to Anton *et al*. (2017), Fuentes *et al*. (2013), Slimestad and Verheul (2005), and Mini (2017), the levels of total phenolic content (TPC) for various tomato cultivars were significantly increased during the subsequent stages of fruit maturity, peaked in pink stage, and then started to gradually decline at the final (RED) stage of fruit maturity.

On the other hand, polyphenol content changed little during the maturation process (Helyes *et al*., 2006). Mature-green harvested tomato fruits showed the lowest ascorbic acid content, increasing levels as the process advances (Getinet *et al*., 2008; Giovanelli *et al*., 1999). The higher alkaloid content in unripe tomatoes is another remarkable difference (e.g., solanine and tomatine). Many studies have focused on decreasing their food levels, considered anti-nutritional factors. Solanine and tomatine are potentially toxic (Kozukue *et al*., 2004; Izawa *et al*., 2010). Limit levels should remain below 200 mg g^{-1} or 100 mg g^{-1}, according to FAO / WHO (1999) and the Nordic health risk assessment (Slanina, 1990), respectively.

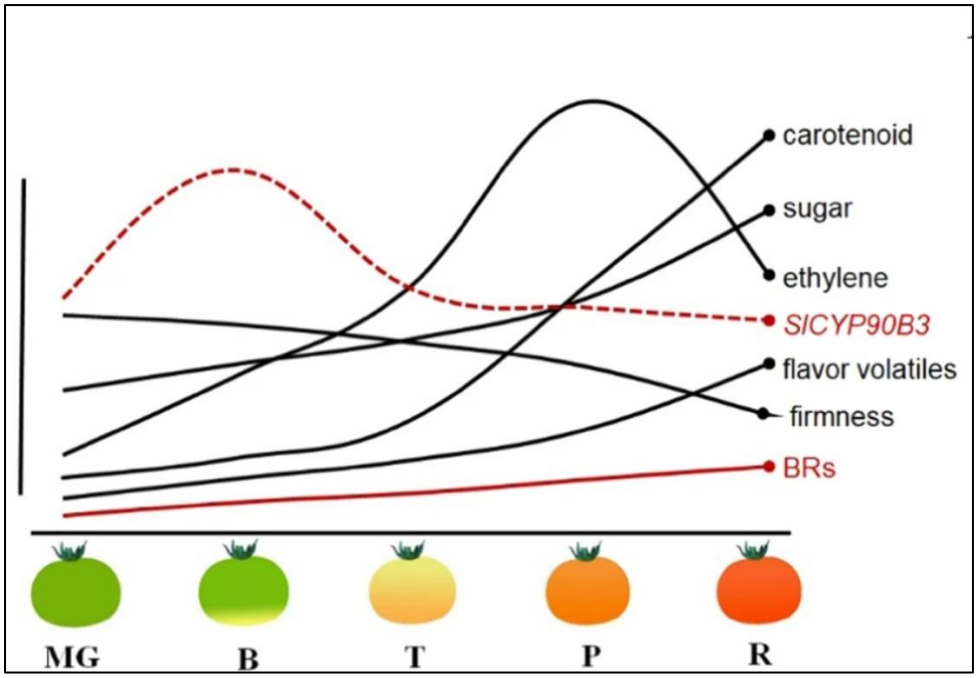

Figure 1.3 - Nutritional in tomato fruit during maturation (MG, mature green; B, breaker; T, turning; P, pink; R, red. (adapted from Songshen *et al.*, 2020)

The EPA estimated that around 31% of all the fresh tomatoes bought by householders were thrown away in the USA. This amount represents approximately 21 tomatoes per person each year. This loss is estimated to cost over 2.3 billion dollars each year. It is not exclusively related to food loss, as it also means the loss of resources such as freshwater, energy, and farming fields. In addition, around 3–7% of the material is lost during tomato processing. This "waste" is commonly called tomato pomace, being mostly made of peels, seeds, and some residual tomato tissue. Despite these by-products being usually discarded, they still are nutrient- and vitamin-enriched sources. (Løvdal *et al.*, 2019; Carillo *et al.*, 2018). Additionally, there is another rejected fraction to consider: the non-harvested green tomatoes, leaves, and roots. Despite the differences mentioned above in the composition of immature tomato fruits, they still have nutritional and functional value that can be put to good use.

The typical composition of tomato pomace on a dry basis, shown in Table 1.1, demonstrates valuable ingredients, supporting valorising these by-products and encouraging industries to transition to renewable bioproducts.

Table 1.1 - Typical composition of tomato pomace on a dry basis (Al-Wandawi *et al*., 1985; Benítez *et al*., 2018; Liadakis *et al*., 1995; Yasmini *et al*., 2019)

Pomace fraction	**Content (% w/w)**	**Components**	**Water content (%)**
Pulp	0-15	Cellulose, hemicellulose, lignin	≈95
Peels	30-60	Cutin, pectin, hemicellulose, cellulose, extractives	≈80
Seeds	35-55	Hemicellulose, cellulose, lignin, lipids	≈60

Table 1.2 shows some experiments regarding the utilisation of Tomato Plant and Tomato Processing Residues during the last decade.

Table 1.2 - Tomato by-product valorisation examples for the production of food ingredients.

Tomato by-product	***Strategies of valorisation as an additive or food ingredient***	***Reference***
Extracts from tomato pomace containing peel fractions and seeds	It can be used due to its antioxidant and antimicrobial capacity, potent lipid oxidation inhibitor, and as an alternative to sodium nitrite in cooked pork sausages.	Garcia Herrera *et al*., 2010; Šoji'c *et al*., 2020
Powders of seeds and peels	It can be utilised as an additive for cheese, bread, and other foods.	Yasmini *et al*.,2019
Tomato pomace meal and tomato seed powder	It can be utilised to make crackers and bread as an ingredient.	Mehta *et al*., 2018
Defatted tomato seeds	It can be used as a suitable medium for cultivating kefir cultures.	Mechmeche *et al*.,2017
Dried and defatted seed	Ferments of these matrices (by *Bacillus subtilis*) can be used to produce hydrolysates with antioxidant and antibacterial activities.	Moayedi *et al*., 2016
Dried peel and seed	These matrices can recover natural antioxidants and edible oil by chemical processes.	Azabou *et al*., 2020
Tomato leaves	Used to extract volatile aroma components	Buttery *et al*., 1987
Tomato leaves	To extract antifungal compounds against plant pathogenic fungi.	Baldwin *et al*., 2000

Tomato by-product	*Strategies of valorisation as an additive or food ingredient*	*Reference*
Tomato pomace (seeds, pulp, and skin)	Used as matrices for solid-state fermentation (by *Aspergillus awamori*) to produce some hydrolytic enzymes (xylanase, exo-polygalacturonase, cellulase and α-amylase).	Umsza-Guez *et al*., 2011
Unripe green tomato	The resulting fermented products (by a consortium of yeast bacteria and lactic acid) can be used as food ingredients with high nutritional value.	Simões *et al*., 2021
Tomato processing by-products	Use these by-products as an efficient source of lycopene extraction (by fermentative processes (solid-state) using *Fusarium solani pisi*).	Azabou *et al*., 2016
Unripe green tomato	Characterising the lactic microbiota of unripe green tomatoes to select LAB strains for use as starter cultures in fermentation processes to forecast the production of food ingredients.	Pereira *et al*., 2021

1.3. Fermentation: Ancient Technique to Preserve Food

Fermentation is a food preservation and transformation technique that relies on microbes' growth and metabolic activities. The metabolites produced by the fermenting organisms limit the growth of spoilage and pathogenic microorganisms during food fermentation and extend the shelf life of perishable produce. Fermentation gives aroma, flavour, texture, and nutritional profile to food in addition to preservation. Although ancient civilisations created fermentation to preserve perishable agricultural produce, now it can thus be considered a helpful approach in improving the products' organoleptic properties, nutrient profile, and probiotic potential, in addition to the preservation function (Motarjemi, 2002). Another advantage could be its contribution to decreasing toxic and anti-nutritional compounds (such as α-tomatine and dihydro tomatine), helping to reduce the potential risk of high alkaloid levels in immature tomato fruits (Kozukue *et al*., 2004; Gupta and Abu-Ghannam, 2012). Various materials, techniques, and microorganisms are used for fermentation. The fermentation can occur spontaneously or be triggered by the addition of specially selected starter cultures. Fermentation is accomplished by decreasing the pH of the food using microbes that convert the carbohydrates in the product into acid. (Hui *et al*., 2004). The fermentation also has many

relevant side contributions: (i) allows the reduction of undesirable compounds, such as naturally occurring anti-nutritional factors, enzyme inhibitors, flatulence factors, tannins and saponins, and undesired intrinsic factors substances that might affect the rate and extent of essential bioconversions. (Xiang *et al*., 2019) (ii) increases the digestibility of products (Hui *et al*., 2004) and (iii) contributes to promoting human health, namely gut health by adding prebiotics, probiotics (Xiang *et al*., 2019) and paraprobiotics and postbiotics, (Cuevas-Gonzales *et al*., 2020) that help preserve and renew the intestine' natural microbiota and modulate immune responses.

There are four main fermentation types: alcoholic (for wine and beer production, predominantly by yeast), acetic acid (for vinegar, ascorbic acid and cellulose production), alkaline (for the production of typical Asian and African products, such as dawadawa, ugba, bikalga, kinema, natto, and thuanao), and lactic acid (LA).

1.3.1. Latic Acid Fermentation

As its name suggests, this fermentation is carried out by lactic acid bacteria (LAB). For decades, Lactic acid bacteria (LAB) fermentation has been found to be applied in the dairy industry, wine and cider production, fermented vegetable products and meat industry (Taskila and Ojamo, 2013). Lactic acid fermentation is a valuable and straightforward technology, low-cost and sustainable process to maintain and improve raw materials' nutritional and sensory properties and extend the shelf-life of fruits and vegetables under sanitary safety conditions (Di Cagno *et al*., 2013). Lactic acid fermentation-derived fermented foods have been produced for thousands of years due to their healthy features, which consumers accept without restriction. LAB is Gram-positive, acid-tolerant, in general non-sporulating, catalase-negative bacteria. LAB's primary function is to produce lactic acid, that is, the acidification of the food.

LAB comprises the Bacteria Kingdom, Phylum Firmicutes and Bacilli Class and Lactobacillales Order, subdivided into six families: *Aerococcaceae*, *Carnobacteriaceae*, *Enterococcaceae*, *Lactobacillaceae*, *Leuconostocaceae* and *Streptococcaceae.* The principal genera of the LAB group include *Lactobacillus*, *Carnobacterium*, *Aerococcus*, *Pediococcus*, *Tetragenococcus*, *Leuconostoc*, *Oenococcus*, *Weisella*, *Enterococcus*, *Lactococcus*, *Streptococcus* and *Vagococcus* (Lahtinen *et al*., 2012).

LAB cultures are generally recognised as safe (GRAS status), playing an essential role in fermentation processes, both utilised in natural fermentation (native microbiota) and controlled fermentation (starter cultures) (Pereira *et al*., 2020). Besides, LAB contributes to the flavour, texture, and nutritional value of fermented foods, through the production of aroma components (Beresford and Cogan,1997; Picon, 2018), production or degradation of exopolysaccharides, lipids and proteins, production of nutritional components such as vitamins, and used as functional cultures, and promoting therapeutic effects and used as probiotics (Bintsis, 2018; Picon, 2018; Wedajo, 2015). In addition, they contribute to the inhibition of spoilage and pathogenic microorganisms and are consequently used as bio-protective cultures (Grattepanche *et al*., 2008)

Traditional fermentation of vegetables depends on the microorganisms found in the raw material and is carried out spontaneously (natural fermentation by indigenous bacteria). On the other hand, starter cultures are used as selected microbial preparations to increase the efficiency of fermentation processes. LAB strains as starter cultures (controlled fermentation) depend on the matrix to be fermented. In this option, selecting specific lactic acid bacteria can improve fermented foods' sensory and nutritional quality. LAB cultures are widely used in fermentation processes, mainly from *Lactobacillus, Leuconostoc, Pediococcus,* and *Lactococcus* (Leroy & De Vuyst, 2004; Di Cagno *et al*., 2013; Montet *et al*., 2014).

Many authors have focused on characterising the lactic microbiota in tomato fruit to select strains with the potential to be used as starter cultures in their fermentation (Di Cagno *et al*., 2009; Mechmeche *et al*., 2017; Santos *et al*., 2019, Pereira *et al*., 2021)

1.3.2. Factors that influence the Latic acid bacteria fermentation

Seven factors influence the growth and activity of LAB in fermenting fruits and vegetables. These include pH, water activity, O_2 concentration, temperature, nutrients, selected starter culture, and inoculum concentration (Lee and Salminen 1995; Ballesteros *et al*. 1999). The pH is critical in preserving and developing the aroma and flavour of many fermented fruits and vegetables like cabbage and olives (Muyanja *et al*., 2003; Rao *et al*., 2004). The majority of Lactic Acid Bacteria prefer near-neutral pH conditions. (Battcock and Azam-Ali 2001). Certain bacteria are acid-tolerant (i.e., *Lactobacillus* and *Streptococcus*) and can survive at reduced pH levels (3.0–4.0) (Ray and Panda 2007). The O_2 requirements vary from species to species. However, unlike many anaerobes,

most LAB strains are not sensitive to O_2 and can grow in their presence and absence. They are aero-tolerant anaerobes (Molenaar *et al*. 2005). Temperature is a critical factor for vegetable fermentation. Most LAB cultures have a temperature optimum between 20 °C to 30 °C; there are some (thermophiles) who prefer high temperatures (50–55 °C) and those with colder temperatures optima (15–20 °C). Most LAB cultures grow best at 18–22 °C (Ray and Panda 2007). Salting is an essential step in vegetable fermentation. LAB can tolerate high salt concentrations during fermentation, generally within 20 to 80 g/l of sodium chloride. This salt tolerance gives them an advantage over less tolerant species and allows LAB fermentation to inhibit the growth of non-desirable organisms (Rao *et al*., 2004). Salt induces plasmolysis in plant cells, releasing mineral salts and nutrients from the vacuole and creating anaerobic conditions for the proper growth of LAB around the submerged product (Gardner *et al*., 2001, Rakin *et al*., 2004, Wouters *et al*., 2013).

LAB requires a high-water activity (0.9 or higher) to survive. A few species can tolerate water activities lower than this, but the yeasts and fungi usually predominate on foods with a lower activity (Ray and Panda 2007). All bacteria require a source of nutrients for metabolism. The fermentative bacteria require either simple sugars such as glucose and fructose or complex carbohydrates such as starch or cellulose (Ray and Panda 2007, Wouters *et al*. 2013).

Using starter cultures is considered a desirable strategy for the industrial production of plant-based fermented products, alternatively to the natural fermentation process. Then, the demand for starter culture is increasing nowadays (Lee *et al*., 2015). The selection of starter crops (allochthonous or indigenous) depends mainly on their competitiveness with the natural flora and the sensory quality of the resultant products (McFeeters 2004). The main selected criteria include: (i) Lack of production of toxic chemicals, (ii) Ability to produce only (L+) lactic acid, (iii) Low or nil production of biogenic amines, (iv) Genetic stability of the species, (v) Rapid brine acidification (vi) Production reproducibility between different batch cultures (vii) Total depletion of fermentable sugars (viii) Resistance to bacteriocins and bacteriophages from natural strains (ix) Potential of strain preservation by drying, freezing, or freeze-drying.

Generally, microbial development, in particular LAB, takes place during several phases: the lag phase, in which microorganisms adapt to the environment; the exponential phase, which corresponds to the period of optimal development of the microbial

population; the stationary phase, in which the number of microorganisms reaches a maximum value and maintained microbial levels; and, finally, cell death (Fig 1.4).

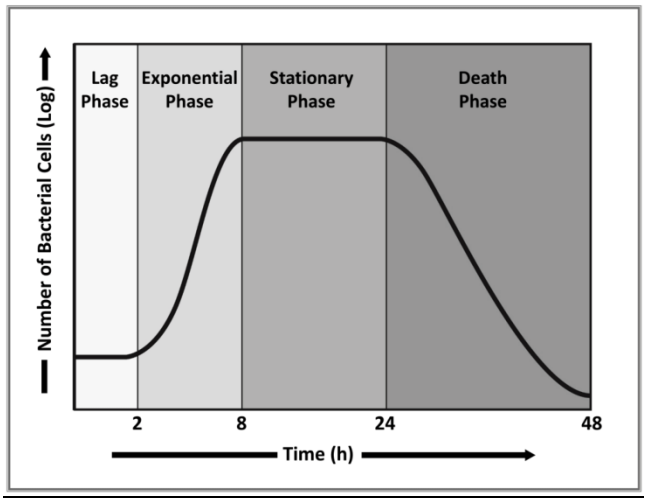

Figure 1.4 - Phases of bacterial growth. Adapted from Garrison & Huigens (2017).

1.4. Functional Foods

The population's awareness of certain foods' beneficial properties led the industry to invest and develop innovative products, such as the so-called "functional foods". In addition to the inherent nutritional effects, these foods may present in their constitution ingredients (such as antioxidants and dietary fibres) or a structure and properties that can be functionally modified after consumption, conferring benefits to the consumer. These beneficial effects must be associated with doses that can be integrated into a regular diet. The formulation of functional foods should reflect and consider the expected behaviour throughout the digestion process, from its initial physical transformation to the absorption of its nutrients (Bornhorst *et al.*, 2016). Furthermore, for a food to be considered functional, its consumption must increase the bioavailability of some nutrients. Accordingly, the total amount of a nutrient released from the food must be absorbed to reach the bloodstream and thus exert its functions in the target organs (Lucas-González *et al.*, 2018). Another possibility is that the food provides components considered beneficial for the consumer's health, such as foods with incorporated probiotic cultures (Gobbetti *et al.*, 2010). The term functional foods first appeared in Japan in 1984, when scientists studied the relationship between nutrition, sensory satisfaction and modulation of physiological systems. They concluded that functional foods demonstrate beneficial physiological effects that reduce the risk of chronic diseases and nutritional benefits (Silveira *et al.*, 2009; Costa & Rosa, 2016; Pomeranz, 2012). Such foods,

designated as Foods for Specific Health Use (FOSHU), carry a seal of approval from the Japanese Ministry of Health and Welfare. Other countries quickly adopted the concept (Costa and Rosa, 2016).

The United States, through the American Dietetic Association (ADA), defines whole, fortified, enriched or enhanced foods as functional foods, assuming the principle of a potentially beneficial effect on health when consumed as part of a balanced diet at adequate levels (Hasler and Brown, 2009).

A critical aspect of functional foods is regulating and declaring their health benefits, which varies markedly between countries. Resolution of the Collegiate Board of Directors RDC No. 2/2002 (Lima, 2019) applies at the national level to the guidelines to be adopted for the safety assessment, registration, and commercialisation of bioactive substances and isolated probiotics with functional and health claims presented as pharmaceutical forms (capsules, tablets, powders, granules, suspensions and solutions). Among the products are carotenoids, phytosterols, flavonoids, phospholipids, organosulfur compounds, polyphenols, and probiotics (Table 1.3).

Table 1.3 - The functional and health property claim in functional foods.

Products	**Compound**	**Claim**
Fatty acids	EPA and EDA	Maintaining healthy levels of triglycerides*
Carotenoids	Lycopene Lutein Zeaxanthin	Antioxidant action that protects cells against free radicals*
Food Fibers	Food Fibers Resistant dextrin Lactulose Partially guar gum Hydrolyzed Polydextrose Beta-glucan Fructooligosaccharide Inulin	Support bowel function* Helps in lowering cholesterol* Intestinal flora balance*

Products	Compound	Claim
	Psyllium Chitosan Phytosterols	 Decreased fat absorption* Reduced absorption of fat and cholesterol* Decreased absorption of cholesterol*
Polyols	Mannitol/ Xylitol/ Sorbitol	Do not produce acids that damage teeth*
Probiotics	Probiotics	The functional or health claim must be proposed by the company and will be evaluated, on a case-by-case basis, based on the definitions and principles established in Resolution no. 1999/18*
Soy Protein	Soy Protein	Cholesterol reduction*

*Its consumption must be associated with a balanced diet and healthy living habits. (Lima, 2019)

Concerns about the impact of food consumption on health and social and environmental consequences have resulted in significant changes at all stages of the food chain in recent years (Falguera *et al*., 2012). As a result, the market for functional foods has shown promise in recent decades (Sanders *et al*., 1999; Corbo *et al*., 2014). Some synthetic food additives, such as antimicrobial agents and antioxidants, are hazardous. Its use is because they prevent the food from spoiling and ensure the quality of organoleptic properties during its shelf life. The consumer preference for fresh, safe, tasty foods with lower levels of sugar, fat, and salt, which can also guarantee desirable physiological effects on health, confirms the demand to reduce the use of such additives (Carocho *et al*., 2015).

In this regard, functional foods play a prominent role, as demonstrated by the growing demand derived from the rising cost of healthcare. Artisan products, functional foods and nutraceuticals can contribute to this scenario. Due to their numerous reported beneficial effects, foods with probiotic cultures can be considered functional foods.

1.4.1. Benefits of probiotics for health

The consumption of probiotics is considered beneficial for health as it prevents infections, improves the performance of the immune system and fights various diseases,

in addition to intestinal diseases, safeguarding the individual's good health. Probiotics have come to be recognised as beneficial for the treatment of diseases in the field of metabolic syndrome (e.g. obesity, diabetes, cardiovascular disease, non-alcoholic fatty liver disease), psychotropic activity through the microbiota-gut-brain axis, and anti-mutagenic or anti-cancer activities (Quigley, 2019; Zoumpopoulou *et al*., 2017).

Probiotics act both at the level of improving the immune system and at the level of improving metabolism. In the case of anti-inflammatory action, they trigger a cascade of reactions through interactions between the mucus membrane and the immune system.

In the case of enteric infections, probiotics can improve the host's defence system against pathogens by promoting mucin production and reducing intestinal permeability. The mucin prevents the penetration of pathogenic organisms and toxic substances or even the production of antimicrobial compounds capable of inhibiting the growth of many food-borne pathogens. These support intestinal barrier function (maintain intestinal impermeability), which is related to various diseases such as irritable bowel syndrome, inflammatory bowel disease and leaky gut and bacterial translocation, consequently leading to liver-related diseases. The production of organic acids by lactic acid cultures, such as lactate and acetate, creates an acidic environment that impairs infections by enteric pathogens. When bacteriocin-producing strains are present, this inhibitory effect can be increased.

More recently, there have been several studies that link the importance of the microbiota-gut-brain axis with the benefits of intestinal function, affirming the ability of the intestinal microbiota to modulate brain development and, consequently, influence emotions and behaviours (Felice & O'Mahony, 2017; Quigley, 2019; Vaikunthanathan *et al*., 2016). Under normal physiological conditions, this axis modulates digestive methods (e.g., motility, secretion), immune function, perception, and emotional response to visceral stimuli. In the intestine, bacteria can produce neuroactive substances that can impact the local level, altering secretion, motility and blood flow, as well as the central nervous system (CNS) (Figure 1.5). The high comorbidity (association of several diseases in the same patient) between psychiatric symptoms associated with stress, such as anxiety, with gastrointestinal disorders, including irritable bowel syndrome and inflammatory bowel disease, is further evidence of the impact of this axis (Felice & O' Mahony, 2017).

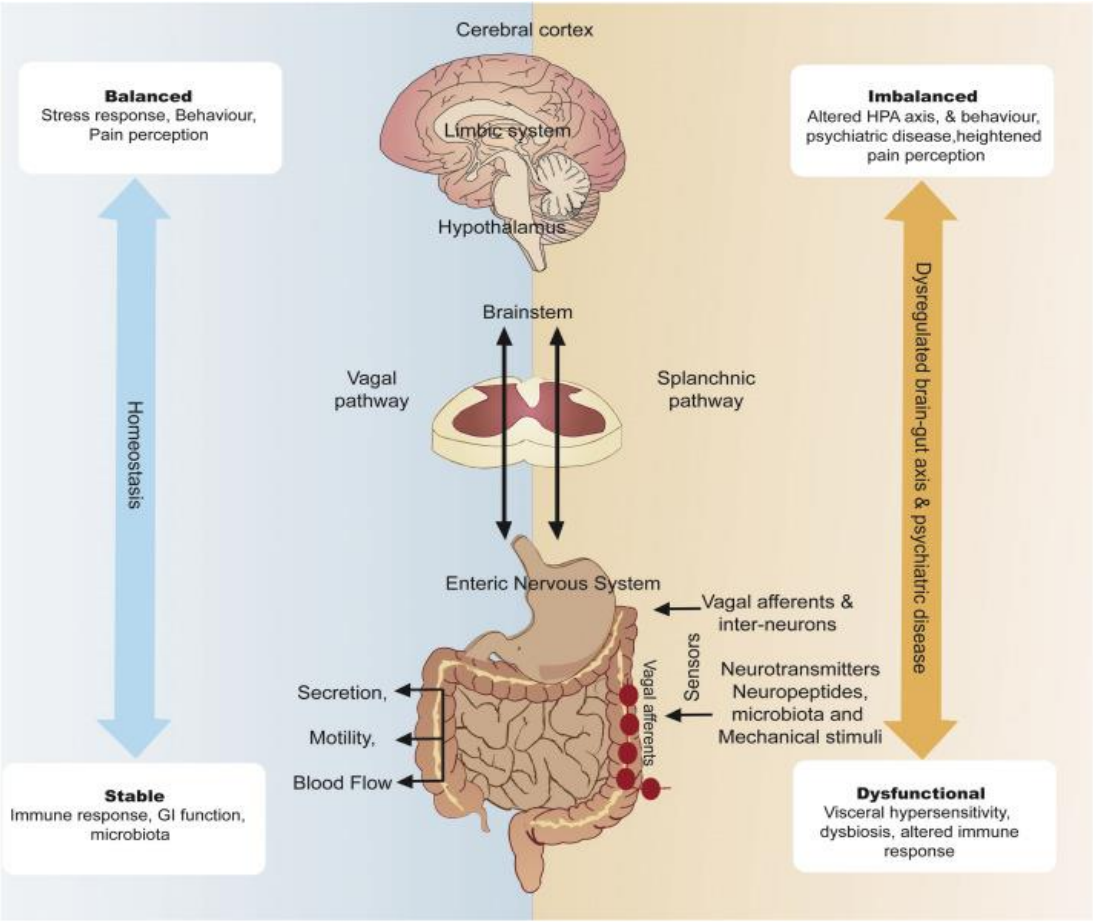

Figure 1.5 - Dysregulated microbiota-gut-brain axis and dysbiosis are associated with central nervous system changes and psychiatric disorders. (Adapted from Felice & O'Mahony, 2017)

Table 1.4 lists some benefits and therapeutic applications associated with probiotics consumption. It should be noted that the beneficial effects and the respective therapeutic applications depend, among other factors, on the probiotic strains considered (Lourens-Hattingh & Viljoen, 2001; Sanders *et al*., 2018).

The significance of the effects of probiotics on humans is poorly understood because most currently available studies were performed in animal models such as rodents and swine (Pirbaglou *et al*., 2016). The results in humans are less consistent and challenging to demonstrate, possibly justified by the fact that the effects of probiotics can be subtle. Their activities can occur at levels that are not easily detectable through stool analysis (Quigley, 2019). Even so, probiotics have been extensively studied to be included in foods that guarantee their viability and remain active at high levels in the final product and during its shelf life (Kok & Hutkins, 2018).

Table 1.4 - Beneficial effects and therapeutic applications of probiotic bacteria in humans. (Adapted from (Lourens-Hattingh & Viljoen, 2001) and (Sanders *et al*., 2018))

Beneficial effects	**Therapeutic applications**
Maintenance of intestinal microflora	Prevention of urogenital infection
Immune system improvement	Constipation relief
Reduction of lactose intolerance	Traveller's Diarrhea Protection
Lowering serum cholesterol levels	Prevention of infant diarrhoea and colic in breastfed babies
anti-carcinogenic activity	Reduction of antibiotic-induced diarrhoea
Improving the nutritional value of food	Prevention of hypercholesterolemia
Reduction in the incidence and duration of common infectious diseases (upper respiratory and gastrointestinal tract)	Colon and bladder cancer protection
	Osteoporosis prevention
	Prevention of atopic dermatitis (in babies)
	Prevention of food hypersensitivity (in babies)
	Prevention of necrotising enterocolitis
	Extending the remission period of ulcerative colitis
	Improve the therapeutic efficacy of antibiotic treatment of bacterial vaginosis

One of these foods is fermented foods, such as yoghurt; these have great acceptance by consumers, have a significant cultural history, and have excellent nutritional value, with yoghurt being one of the best-known fermented dairy products for thousands of years (Ashraf & Shah, 2011; Sanders *et al*., 2018). However, other foods have been formulated, such as cheese, cereal-based products such as oat bars, nuts, natural juices and some vegetables, such as olives preserved in brine (Lavermicocca, 2006). In the case of fermented functional foods, the health benefits can be expressed directly through the interactions of the host with the ingested live microorganisms (probiotic effect) or indirectly as a result of the ingestion of microbial metabolites synthesised during fermentation (biogenic effect) (Gobbetti *et al*., 2010).

There is a sustained increase in the demand for non-dairy beverages of high functional value, fresh, nutritive, healthy, and appetising foods and drinks. Popular trends towards vegetarianism and veganism and the prevalence of lactose intolerance and allergy to cow's milk proteins are in accelerated development. In this context, single-fruit, blend smoothies, or fruit juices that LAB can ferment constitute a promising alternative to supply the mentioned needs and promote fruit consumption (Nazhand *et al*., 2020; Ruiz Rodríguez, 2018; Szutowska, 2020).

1.4.2. Fermented Products as a Source of Probiotics

The high valorisation of fermented products lies in their potential as a source of probiotics. Probiotics are live microorganisms that confer a health benefit on the host when administered in adequate amounts. The microorganisms approved so far by ANVISA (Lima, 2019) are shown in Table 1.5.

Table 1.5 - Microorganisms with proven probiotic properties.

Lactobacillus	**Bifidobacterium**	**Enterococcus**
L. acidophilus	*B. bifidum*	*E. faecium*
L. casei shitota	*B. animallis (including the subspecies B. lactis)*	
L. casei rhamnosus variety	*B. longum*	
L. casei defensive variety		
L. paracasei		
L. lactis		

Adapted from National Health Surveillance Agency (Lima, 2019).

Although a substantial number of microbial species have been reported to exhibit potential probiotic properties, established after *in vitro* and preclinical research and/or large-scale clinical trials, only those more documented and robust strains may reach the market (Foligné *et al*., 2013). However, the list of probiotic strains is relatively short. The primary examples concern strains offered by the dairy industry and some scientific groups. The most common probiotics are lactic acid bacteria (LAB) belonging to the genera *Lactobacillus* and *Bifidobacterium*. Also included with less representativeness are *leuconostoc, pediococci, lactococci, enterococci* and *streptococci* (Foligné et al., 2013; Holzapfel and Wood, 2014). It is generally accepted that, except for *enterococci* in some

countries, due to safety concerns concerning the ability to transfer antibiotic-resistant genes, LAB are rarely pathogenic for humans and animals (Holzapfel; Schillinger, 2002).

LAB cultures have been extensively used (i) as starter cultures; (ii) as probiotics; and (iii) in the production of valuable compounds (i.e., nutraceuticals) due to their versatile metabolism (Emerenini, 2013; Ruiz Rodríguez *et al*., 2019). Once LAB cultures have been ingested orally with food, they must first survive transit through the stomach. The secretion of gastric acid constitutes a primary hurdle to overcome before reaching the intestinal tract (Dunne *et al*. 1999). Therefore, the resistance and tolerance level of any LAB to be used as a putative probiotic strain must be evaluated *in vitro* against the typical components of gastric juice, for instance, low pH, bile, pepsin and pancreatin, to mimic the conditions *in vivo* in the GI tract (Conway *et al*., 1987; Dunne *et al*., 1999). Additionally, the probiotic must survive food-processing stressors, such as fermentation, freeze-drying, variations in temperature, pH, and oxidative and osmotic stress during storage (Frakolaki *et al*., 2021). The probiotic species' high viability during food storage is critical and required. Specifically, the minimum concentration of probiotics is approximately 10^6–10^7 CFU/ml at the time of food product consumption. (Terpou *et al*., 2019; Kazakos *et al*., 2020). It has been established that the survival of probiotic strains in fermented foods is restricted mainly by salt and other factors, including oxygen and temperature (Champagne *et al*., 2005). Therefore, obtaining starter cultures from the *Lactobacillus* strains that often exist naturally in fermented vegetables, and testing these for potential probiotic characteristics, may make it possible to overcome problems of survival.

The approach of valorising unripe tomatoes by lactic fermentation with the addition of starter culture to produce a fermented ingredient was previously studied. In a previous study, the production of food ingredients by fermentation of immature tomatoes was successfully tested by adding two strains of lactic acid bacteria (LAB) as starter cultures, namely *Weissella paramesenteroides* and *Lactiplantibacillus plantarum*. The present work aims to complement this valorisation strategy and address the following issues: selecting the best ingredient according to the added input, re-evaluating the probiotic potential of LAB strains, and formulating a healthy sauce based on fermented green tomatoes. The study was divided into three phases:

- To select the best option for producing a nutritionally valuable sensory ingredient, use LAB strains as starters (*Weissella paramesenteroides* and *Lactiplantibacillus plantarum*) to characterise two fermented tomatoes.
- To assess the probiotic potential of the mentioned strains by sequential *in vitro* digestion simulation model with the presence of digestive enzymes.
- Establish a sensory-acceptable sauce formulation and evaluate its stability throughout refrigerated storage for 21 days (5 °C).

EXPERIMENTAL PART

2. MATERIAL AND METHODS

2.1. Plant material and sample preparation

2.1.1. Plant material

Immature tomato *cv* H1015 fruit (a standard variety for tomato-paste production) was supplied by the Competence Centre for Industry Tomato (Centro de Competencias Tomate de Indústria, CCTI). After mechanical harvesting of mature fruit, immature fruit was manually collected from the fields located in the Ribatejo region (municipalities of Azambuja, Benavente, Cartaxo and Vila Franca de Xira) and transported to the laboratory facilities of the Technology and Innovation Unit (UTI) of the National Institute of Agrarian and Veterinary Research (INIAV). Upon arrival, the fruit was sorted into two categories based on their surface colour (Figure 2.1): green fruit (identified as green category, G) and fruit with multiple tones of green, red, and yellow (identified as intermediate category, I). All fruit were washed under running tap water, dried with absorbent paper, packaged according to the colour category, and stored at -20±1 °C (Cryocell Aralab, Rio de Mouro, Portugal) until use.

Figure 2.1 - Tomato fruit appearance, according to the category, after freezing (-20±1 °C).

2.1.2. Pulp Processing

Pulp processing was conducted in a sanitised room using sanitised apparatuses to prevent contamination during processing. Immature-tomato pulps were prepared from previously tawed fruit at 5 °C (24 hours). Pulps were established from fruit from the G

and I category, mixed in a 1:1 ratio (w:w) and homogenised with 1.5% NaCl solution in a Robot Termomix (Vorwerk, Germany), set at maximum speed for 1 min. After homogenisation, pulps were distributed in 1 L Schoot flasks (Figure 2.2). The fruit category mixture proportion was established according to the relative proportions of each category observed in the fields.

Figure 2.2 - Immature tomato pulp appearance

2.2. Microbial Methods

All microbiological determinations were conducted at the UTI-INIAV Microbiology laboratory according to the laboratory guidelines of ISO 7218:2007 and ISO 6887-1:2017 for sample preparation and dilutions.

2.2.1. Starter LAB cultures

Two strains of pure LAB cultures with *in vitro* probiotic potential, one from the INIAV collection (*Weissella paramesenteroides*; id.: C1090) and another isolated from immature tomato lactic microbiota (*Lactiplantibacillus plantarum*; id.: LAB97) (Pereira *et al*., 2021), were tested as starters in the fermentation of immature tomato pulp.

C1090 and LAB97 strains were activated by double culture in de Man, Rogosa and Sharpe (MRS) broth (Biokar Diagnostics, Allone, France) incubated at 30 °C for 24 h. After activation, the LAB strains were cultured in MRS agar (Biokar Diagnostics, Allone, France) and incubated at 30 °C for 24 h to ensure a pure culture condition. Pure colonies were isolated to prepare the cell suspensions used for the study. The suspensions were dispersed in 0.85% NaCl solution (Sigma-Aldrich, St. Louis, Missouri, USA), adjusted

to MacFarland Standard 5 (corresponding to a cell density of *ca*. 10^9 CFU/ml) and distributed into fermentation flasks to achieve a final concentration of 10^8 CFU/ml.

2.2.2. Lactic Acid Bacteria Counts

LAB counts were determined according to ISO 15214:1998. The viable colony counts (LogCFU/ml) from each test condition were determined using the pour-plate method (MRS agar) with the appropriate decimal dilutions after incubation at 30 °C for 72 h, and counts were estimated as:

$$N = \frac{\sum C}{V \times 1.1 \times d} \qquad \text{Equation 2.1}$$

N is the number of CFU in the sample, C is the sum of the colonies counted on two plates with successive decimal dilutions (with a minimum of 10 colonies), V is the inoculum volume (ml), and d is the dilution rate relative to the first dilution.

2.2.3. Yeast and Mould Counts

The yeast and mould count was performed according to ISO 21527-1:2008, using dichloran Rose-Bengal Chlortetracycline Agar (DRBC) (Biokar Diagnostics, France). After incubation under aerobic conditions at 25 °C for five days, the yeast and mould count was assessed according to Equation 2.1.

2.2.4. Total Microbial Counts

The total microbial count was conducted according to ISO 4833-1:2013(E), using plate count agar (PCA) (Plate Count Agar; Biokar Diagnostics, France). After incubation at 37 °C for 24-48 hours, the total microbial count was calculated according to Equation 2.2. This measurement is represented by the number of colony-forming bacterial units (CFU) per gram (or millilitre) in the sample

$$\text{Calculate CFU/ml} = \frac{\text{number of colonies} \times \text{dilution factor}}{\text{Volume of culture plated}} \qquad \text{Equation 2.2}$$

2.3. Physical-Chemical parameters

2.3.1. pH

The pH was determined using a pH meter (Crison micro pH 2001, Spain), previously calibrated at room temperature, with pH buffer solutions 4 and 7 (Carlo Erba Reagents, Spain). In this study, the mean pH values resulted from 2 determinations per sample.

2.3.2. Total Acidity (TA)

Total Acidity (TA) was determined according to NP–1421 (1977), with slight modifications. 5 g of immature tomato pulp was mixed with distilled water to a final volume of 50 ml (volumetric flask). The solution was filtered (Whatman nº 41), and 10 ml aliquots were titrated under continuous agitation with NaOH 0.1 M (Honeywell®, USA) to pH = 8.2, using an automatic titrator (Metrohm 665 Dosimat). The spent NaOH volume was recorded. TA calculation was achieved by equation 2.3. The mean values of TA are expressed in grams of lactic acid per 100 g of fresh weight (g LA/100 g FW).

$$TA = \frac{(V_{total} \times V_{NaOH})/V_{titrate}}{\text{Weight of Sample}} \times 0.1 \times 0.090 \times 100 \qquad \text{Equation 2.3}$$

with V_{total} corresponding to the total sample volume (in ml), V_{NaOH} to the spent volume of NaOH (in ml), $V_{titrate}$ to the analysed aliquot volume (in ml), 0.1 corresponding to the normality of the NaOH solution and 0.090 to the conversion factor to lactic acid.

2.3.3. Solid Soluble Content (SSC)

The Solid Soluble Content (SSC) was determined using a digital refractometer (Atago Palette PR-201, Tokyo, Japan), previously calibrated with distilled water. In this study, the mean values resulted from 2 determinations per sample and were expressed by percentage weight (% w/w; °Brix).

2.3.4. CIELab Colour

The colour of the unripe green tomato pulps was evaluated in the CIELab system (Illuminant C), using the Minolta Chroma Meter CR-300 colourimeter (Osaka, Japan), calibrated with a white reference standard (L* = 97.10; a* = 0.19; b* = 1.95). The CIE

system describes colour by three coordinates: L*, a*, and b*. (CIE,2004, HunterLab, 2001).

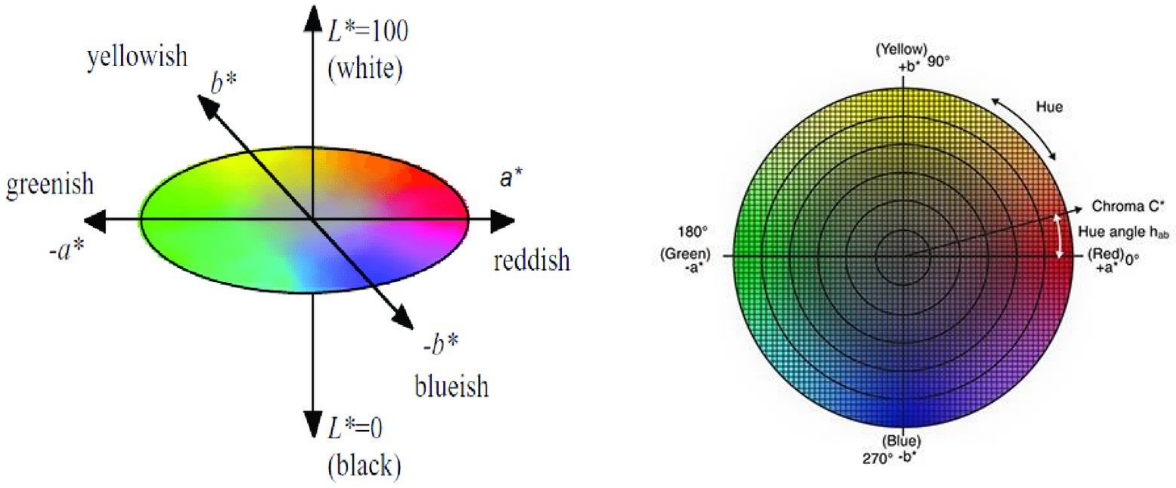

Figure 2.3 - a) Illustration. CIELAB colour space. b) Model of the CIELab spatial chromaticity system, with an illustration of the coordinates L*, a* and b*, as well as the parameters C* and ^{o}h. Taken from Mouw (2018).

The L* value represents the luminosity and depicts the variation between black (L* = 0) and white (L* = 100). The a* value expresses the variation between red (a* = +60) and green (a* = -60), while the b* value is the variation between yellow (b* = +60) and blue (b* = -60). For each sample, triplicates were taken. From the CIELab parameters, the colour saturation or chromaticity (C*, equation 2.4), hue (°h, equation 2.5), whiteness index (WI, equation 2.6) and total colour difference (TCD, equation 2.7 and Table 2.1) were calculated. Figure 2.3 illustrates a schematic of the CIELab spatial system model.

$$C^* = \sqrt{a^{*2} + b^{*2}} \qquad \text{Eq – 2.4}$$

$$°h = \frac{\tan^{-1}\left(\frac{b^*}{a^*}\right)}{6.2832} \times 360, \qquad \text{if } a^* > 0 \text{ and } b^* > 0 \qquad \text{Eq – 2.5}$$

$$°h = 180 + \frac{\tan^{-1}\left(\frac{b^*}{a^*}\right)}{6{,}2832} \times 360, \qquad \text{if } a^* < 0$$

$$°h = 360 + \frac{\tan^{-1}\left(\frac{b^*}{a^*}\right)}{6{,}2832} \times 360, \qquad \text{if } a^* > 0 \text{ and } b^* < 0$$

$$WI = 100 - \sqrt{(100 - L^*)^2 + a^{*2} + b^{*2}} \qquad \text{Eq – 2.6}$$

$$TCD = \sqrt{(L^* - L_0^*)^2 + (a^* - a_0^*)^2 + (b^* - b_0^*)^2} \quad \text{Eq – 2.7}$$

Table 2.1 - Detailed classification of the levels of differences obtained through the TCD, adapted from Allegretti *et al.*, 2009.

	Colour Changes (TCD)	**Description**
1	TCD < **0.2**	No noticeable
2	0.2 ≤ TCD < **2**	Small difference
3	2 ≤ TCD < **3**	Colour differences noticeable at high-quality screen
4	3 ≤ TCD < **6**	Colour differences noticeable in middle-quality screen
5	6 ≤ TCD < **12**	Great difference
6	TCD ≥ **12**	Different colours

2.3.5. Total Phenolic Content (TPC)

Samples were extracted by mixing 2.5 g of immature green tomato pulp with 10 ml of methanol (Honeywell®, USA) and subsequently homogenised (13 rpm × 30 s; Polytron Ultra–Turrax T 25 basic, IKA–Werke). After homogenisation, the mixture was extracted in an ultrasonic bath (Sotel Branson 2200 Ultrasonic Cleaner) for 10 minutes and centrifuged for 20 minutes at 7000 rpm at 4 °C. (Sigma laborzentrifugen 2K15, Osterode am Harz, Germany). The supernatant was collected in Falcon tubes and immediately analysed or frozen at -20 °C until analysis (Large Upright AEG OKO_ARCTIS Freezer).

With some modifications, the total phenolic content (TPC) was determined using the colourimetric reaction promoted by the Folin-Ciocalteu reagent, as described by Swain & Hillis (1959). the reaction was developed as follows: in test tubes, 2400 µL of distilled water, 150 µL of the methanolic extracts (or methanol as a blank), and 150 µL

of Folin-Ciocalteu reagent 0.25 M (Sigma-Aldrich®, USA) were mixed. The mixture was stirred, and after 3 minutes, 300 μL of sodium carbonate 1 M (Merck Millipore®, USA) was added. The reaction was allowed to develop for 2 h under dark conditions (room temperature). Spectrophotometric readings were taken at $\lambda = 725$ nm (Spectrophotometer Jas.co V-530 UV/Vis, Japan). The total phenolic content was expressed as mg gallic acid equivalents per 100 g of fresh tissue (mg GAE.100 g-1), quantified from an external standard curve (0.004-0.600 $mg.ml^{-1}$).

2.3.6. Antioxidant Capacity (AOx)

Using the same methanolic extracts as for TPC determination, the antioxidant capacity (AOx) was determined using DPPH and FRAP methods.

DPPH method

The determination of AOx using the DPPH method was based on the procedures described by Arnao *et al*. (2001) and Brand-Williams *et al*. (1995), with some modifications. This method uses the stable free radical of DPPH* nitrogen (2,2-diphenyl-1-picrilhydrazil), whose purple colour turns yellow after its reduction in the presence of antioxidant compounds (Alves *et al*., 2010).

The DPPH radical (TCI Chemicals) was previously diluted in methanol (1:4.5, v:v) to an initial absorbance of 1.10 ± 0.02 at $\lambda = 580$ nm. The reaction mixture was prepared by mixing 150 μL of extract (or methanol as a blank) with 2850 μL of the prepared DPPH solution and incubated at room temperature for 2 h in dark conditions. After this period, the absorbance reduction was measured spectrophotometrically at $\lambda = 580$ nm (Spectrophotometer Jas.co V-530 UV/Vis, Japan). A standard curve was developed using Trolox (Sigma-Aldrich®, USA) as standard (82–10000 μM) and following the same assay. The results were expressed in μM of Trolox equivalents per 100 g of fresh wright (μM TEAC/100 g FW)

FRAP method

Pulido *et al*. (2000) described the FRAP method (Ferric Reducing Antioxidant Power) as an alternative to determine the antioxidant capacity through the reduction of iron in biological fluids and aqueous solutions of pure compounds (Fig. 2.4).

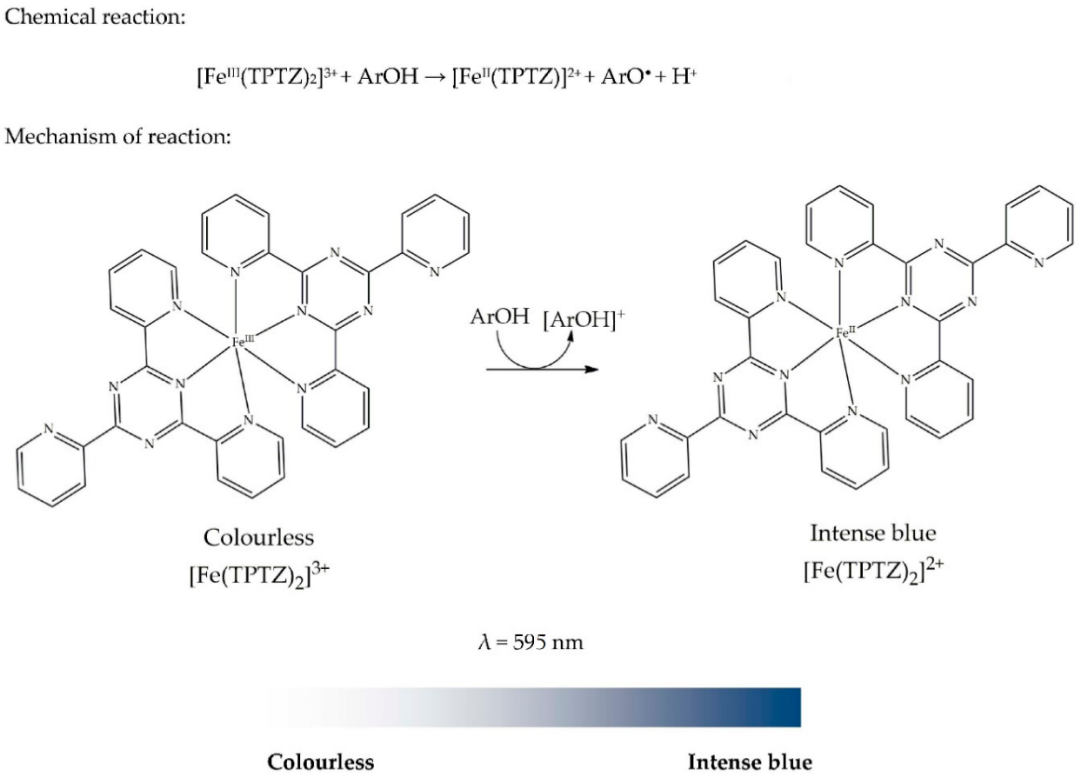

Figure 2.4 - The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power". Adapted from Benzie et al., 1996.

The FRAP method's evaluation of the total antioxidant capacity was adapted from Rufino *et al.* (2006). The sample AOx was determined using a mixture of 2.7 mL of Ferric Reducing Antioxidant Power (FRAP) reagent, 90 µL of the sample methanolic extract (or methanol as a blank), and 270 µL of distilled water. The mixture was incubated (37°C × 30 min), and the absorbance was read at 595 nm. A standard curve using Trolox was developed (82–10000 µM). The antioxidant activity was expressed as µmol of Trolox per 100 grams of fresh weight (µmol TEAC 100 g^{-1} FW) (Trolox equivalent).

2.3.7. HPLC profiles

Organic Acid profile

The organic acids profile and quantification were performed by high-performance liquid chromatography (HPLC) coupled to a photodiode array detector (PDA) in a Waters HPLC system (Alliance 2690, 996 PDA and column thermostat JetStream 2 plus, Milford, MA, US), according to Panda *et al.* 2022, with modifications. Organic acids

extraction was done by weighting 3 g of the sample accurately, followed by adding 0.05 M phosphate buffer at pH 2.8 to a total volume of 13 mL. After homogenization in polytron (Ika, Ultra-Turrax T25, Germany), the mixture was extracted in an ultrasonic bath (Sotel Branson 2200 Ultrasonic Cleaner) for 10 minutes and centrifuged for 20 minutes at 4500 rpm at four °C. (Sigma, 2K15, Germany). The supernatant was filtered into an identified vial through a syringe filter (Filter Lab, Barcelona, Spain).

Organic acids were separated on an ion-exclusion column (Rezex™ ROA, 300 x 7.8 mm, 8 µm particle size, Phenomenex Torrance, CA, US) at 25 °C in isocratic mode with a 0.01 M sulfuric acid mobile phase for 30 min at a flow rate of 0.5 ml/min and 10 µl injection volumes. Solvents were made with ultrapure water (Millipore, model Milli-Q 7000, USA), filtered under vacuum (pump GAST, model DOA-P104-BN, USA) with 0.45 µm nylon membrane (Filter Lab, Barcelona, Spain) and ultrasound degassed for at least 25 minutes. Organic acid identification was made at 210 nm wavelength by comparison of retention time and UV spectrum of the organic acid standards. The peak areas were quantified and processed with version 5.0 of the Empower Pro 2002 Software (Waters Milford, USA) by comparison to the calibration of mix organic acid standards from 20 to 2000 µg/ml for citric, tartaric, succinic, malic and formic acids, from 2 to 200 µg/ml for oxalic acid, from 18 to 1300 µg/ml to ascorbic acid, from 18 to 1820 µg/ml to lactic acid and from 10 to 760 µg/ml to acetic acid standard. Table 2.2 shows the range of Limit of detection and limit of quantification for each organic acid. The limits were calculated based on the standard deviation (Sy) and the slope of the calibration curve (S) according to the formulas: DL = 3.3(Sy/S) and WL = 10(Sy/S).

Table 2.2 - Ranges of detection and quantification for each organic acid

Organic Acid	**Limit of detection (mg/100g)**	**Limit of Qualification (mg/100g)**
Oxalic Acid	4.3	12.9
Citric Acid	26.5	80.4
Tartaric Acid	31.2	94.6
Ascorbic Acid	22.4	67.8
Lactic Acid	65.2	197.7
Succinic Acid	43.8	132.7
Acetic Acid	9.1	27.5
Malic Acid	33.9	102.6
Formic Acid	74.5	225.6

Phenolic profile

The phenolic profile and quantification were performed by HPLC-PDA in the same Waters HPLC system described above, according to Petitjean-Freytet *et al.* 1991, with minor modifications. From the methanolic extracts (as described in 2.3.5), the phenolic compounds were separated on Synergi Hydro 4 µm RP 250 × 4.6 mm column (Phenomenex Torrance, CA, US) at 25 °C. This column was operating in gradient mode with two mobile phases: water (adjusted to pH 2.3 with formic acid) as solvent A and acetonitrile: water (80:20) (v:v) (adjusted to pH 2.3) as solvent B. The gradient system begins with 88/12 (VA/VB), being 85/5 at 5 min, 170/30 at 30min, 50/50 at 35 min, 30/70 from 40 to 45 min, and 88/12 at 60 min, followed by 5 min of column stabilization, the flow rate was 1 ml/min, and using 20 µl of injection volume. Solvents were filtered and ultrasound degassed. Commercial standards were used for peak identification by comparing respective retention times and UV–VIS spectra. Quantifying phenolic compounds was based on a developed external standard curve using mixed standards solutions in the range of 5 to 150 µg/ml. Table 2.3 displays the integrated wavelength for phenolic compounds and the limit of detection and quantification of the compounds determined in mg per 100 g of sample.

Table 2.3 – Wavelength and ranges of detection and quantification for each phenolic compound

Phenolic compound	**Wavelength (nm)**	**Limit of Detection (mg/100g)**	**Limit of Quantification (mg/100g)**
Catechin	280	13.9	20.1
Chlorogenic acid	325	6.6	20.1
Hydroxybenzoic acid	280	4	12.2
Vanillic Acid	280	4.7	14.3
Caffeic Acid	325	6.1	18.5
Syringic acid	280	3.6	10.8
Coumaric acid	325	6.7	20.3
Rutin	340	7.8	23.7
Ferulic acid	325	5.7	17.2
Naringin	280	4.8	14.5
Naringenin	280	88.4	268.0
Quercetin	340	22.2	67.3
Kaempferol	340	128.0	387.9

2.4. Sensory Analysis

A panel of 16 trained panellists conformed to ISO 8586-1 (1993), gathered adequate conditions in compliance with ISO 13299 (1995), and identified and distinguished sensory attributes, such as colour, appearance, taste/flavour, aroma, consistency, and acceptance. Evaluations were scored based on a 9-point hedonic scale, with 1 representing the lowest score (dislike very much) and 9 the highest score (like very much) (Lim, 2011). In a test room intended for sensorial analysis, with individual tasting cabins illuminated with white light, samples were served to the panellists on glass cups marked with three-digit code numbers and presented in a randomised order. Panellists are also asked to enclose any relevant comments.

2.5. Statistical Evaluation

Data were subjected to Multi- or One-Way ANOVA, and the means were compared using Tukey Honestly Significant Difference (HSD) test ($p = 0.05$). Pearson correlation coefficients were also generated between the studied responses.

3. THE PROBIOTIC CAPACITY OF FERMENTED TOMATOES AS AN INGREDIENT

3.1. Probiotic potential assessment of two LAB cultures

3.1.1. Objective

As starters, pure LAB cultures of *Lactiplantibacillus plantarum* (LAB97, isolated from the microbiota of immature tomato) and *Weissella paramesenteroides* (C1090; INIAV collection) exhibited the ability to ferment green tomato pulp to produce a food ingredient. A previous study demonstrated the *in vitro* tolerance of both strains to adverse pH conditions and the presence of bile salts as evidence of their probiotic potential.

- This trial intended to confirm the probiotic character of *Lactiplantibacillus plantarum* (LAB97) and *Weissella paramesenteroides* (C1090) by developing an accurate static *in vitro* simulation model of gastrointestinal food digestion, which combines the sequential digestion steps and the presence of digestive enzymes.

3.1.2. Experimental design

In vitro digestion models aim to simulate the physiological conditions of the upper gastrointestinal tract, namely the oral, gastric, and small intestinal stages, and can be performed by static or dynamic methods. Due to their simplicity, static models, which use a constant ratio of food to enzymes and electrolytes and a specific pH for each digestive phase, have been widely used and have shown much usefulness in predicting the results of in vivo digestion. (Sanchón et al., 2018)

Digestion involves the exposure of the samples (LAB cell suspensions) to three successive digestive phases: oral, gastric, and intestinal, summarised in Figure 3.1. The experimental conditions established for the static digestion model developed, including the LAB cell suspensions and the digestion procedures (such as pH, digestion time, and enzymatic activity), were based on the methods described by Palencia *et al.,* 2008 with some modifications.

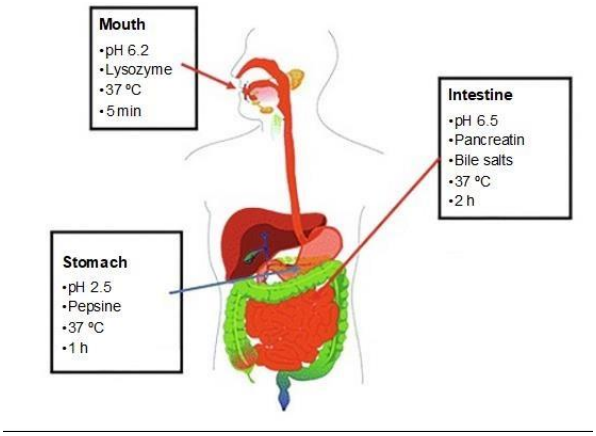

Figure 3.1 - Scheme of digestion steps and their conditions.

Sample preparation: Each LAB strain (pure cultures stored at -20 °C; 100 μl) was grown in De Man Rogosa Sharpe (MRS) medium (10 ml) for 24 h under 37 ºC (anaerobic conditions) until reaching the late exponential phase (approximately 10^9 CFU ml^{-1}). Next, cells from each culture (350 μl) were diluted in 35 ml of MRS to continue cell activation for the 16 h period at 37 °C. The suspensions were then centrifuged (5000×g for 15 min at 4 °C), and the corresponding pellet was resuspended in 35 ml of phosphate-saline buffer (PBS) (consisting of 8.0 g/l NaCl, 0.2 g/l KH_2PO_4, 1.15 g/l Na_2HPO_4 at pH 7.2). This process was repeated twice. After the final centrifugation, the pellets were resuspended in simulated salivary fluid (SSF). The solution was thoroughly mixed with a vortex mixer (Prolab, São Paulo, Brazil) before being transferred to the sterile 100 ml flasks. The flasks were placed in a 37 °C water bath for 20 minutes before starting the first digestion step (t_0).

Stock solutions of digestion fluids: All gastrointestinal solutions were prepared fresh daily according to the protocols, and the composition and pH condition of the simulated fluids are shown in Table 3.1.

Table 3.1 - Composition, concentration, and pH values of each simulated fluid

Stock solution	**Phase**	**Composition and pH**
Simulated salivary fluid (SSF)	Oral	6.2 g/l NaCl; 2.2 g/l KCl; 0.22 g/l $CaCl_2$; 1.2 g/l NaHCO3; 0.01% (m/v) lysozyme at pH 6.2
Simulated gastric fluid (SGF)	Stomach	6.2 g/l NaCl; 2.2 g/l KCl; 0.22 g/l $CaCl_2$; 1.2 g/l $NaHCO_3$; 0.3% (m/v) pepsin at pH 5
Simulated intestinal fluid (SIF)	Intestine	5.0 g/l NaCl; 0.6 g/l KCl; 0.3 g/l $CaCl_2$; 0.45% (m/v) bile salts; 0.1% (m/v) pancreatin at pH 8

Digestion procedures: The conditions for each phase of the simulation of gastrointestinal food digestion were kept constant and performed in triplicate. (Fig. 3.2) In the oral phase, 5 ml of simulated salivary fluid (SSF) was added to 35 ml of each strain in MRS broth. The exposure of the sample to salivary lysozyme (≧40000 U/mg; Sigma-Aldrich) lasted 5 min at pH 6.2. Three ml of simulated gastric fluid (SGF) with pepsin (2000 U/g; Sigma-Aldrich) was added to the resulting suspensions from the oral phase to simulate the gastric conditions. The oral bolus was then incubated at 37 °C under stirring (50 rpm, Metabolic Water Bath Dubnoff MA-095, Marconi, Piracicaba, Brazil) at pH 2.5 (adjusted with HCl (1 M)) for 1 h. Afterwards, the pH of the gastric chyme was adjusted to 6.5 by adding NaOH (1 M), and 4 ml of simulated intestinal fluid (SIF) with pancreatin (≧ 3 USP; Sigma-Aldrich) and bile salts (Sigma-Aldrich) were added to the samples. The suspensions were incubated at 37 °C under stirring (50 rpm) at pH 6.5 for two hours.

In parallel, cell suspensions of each strain were kept in PBS (same proportions) and subjected to the same procedures, except for the addition of enzymes, bile salts, and pH adjustments as a control (Id: Ctrl). LAB82 was performed as a reference sample as it is a strain known for its sensitivity to low pH conditions and the presence of bile salts. In the initial cell suspension (t_0) and at the end of each digestion step (t_S and t_F for gastric and intestinal steps, respectively), cell viability was monitored by LAB plate counts, according to ISO 15214:1998. This procedure was performed in triplicate for each tested strain.

The survival rate was calculated as follows:

$$\text{Survival rate } (\%) = \left(1 - \frac{\text{LAB counts at } t_0 - \text{LAB counts at } t_F}{\text{LAB counts at } t_0}\right) \times 100 \qquad \text{Eq\_2.8}$$

$$\text{Survival rate (\%)} = \frac{\text{LAB counts at } t_F}{\text{LAB counts at } t_0} \times 100 \qquad \text{Eq\_2.9}$$

LAB counts at t_F represent the total viable counts (CFU/ml) for each strain at the final incubation time in SIF, and LAB counts at t_0 represent the total viable counts at the initial time (t_0).

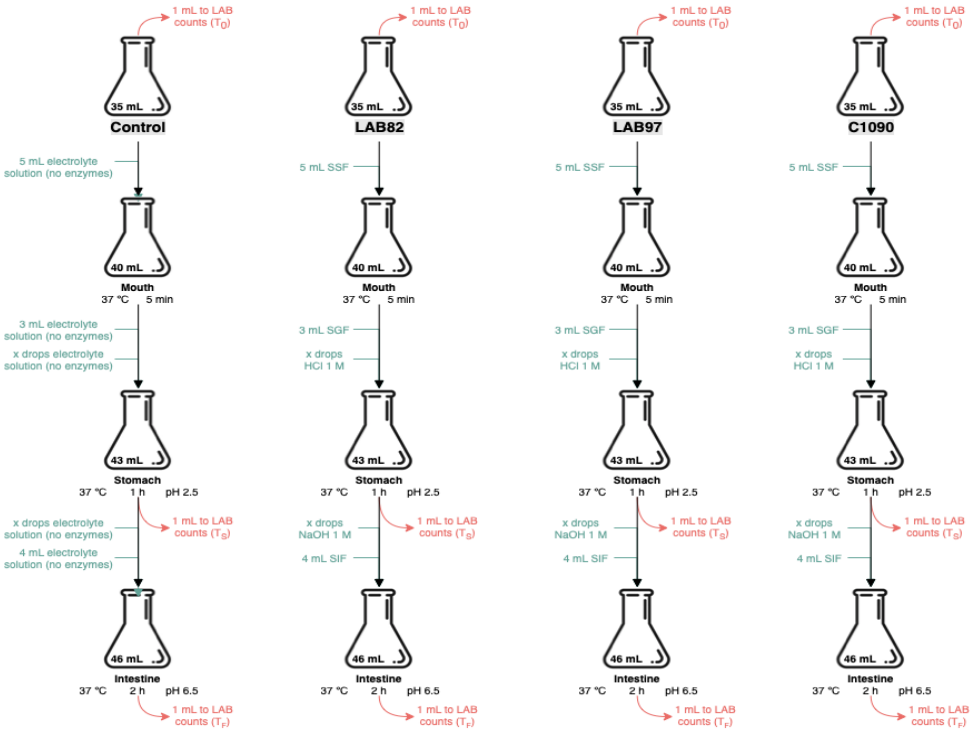

Figure 3.2 - Static digestion model followed for testing the probiotic potential of LAB cultures (LAB 97 and C1090) and control samples (LAB82 and Ctrl).

3.1.3. Results and discussion

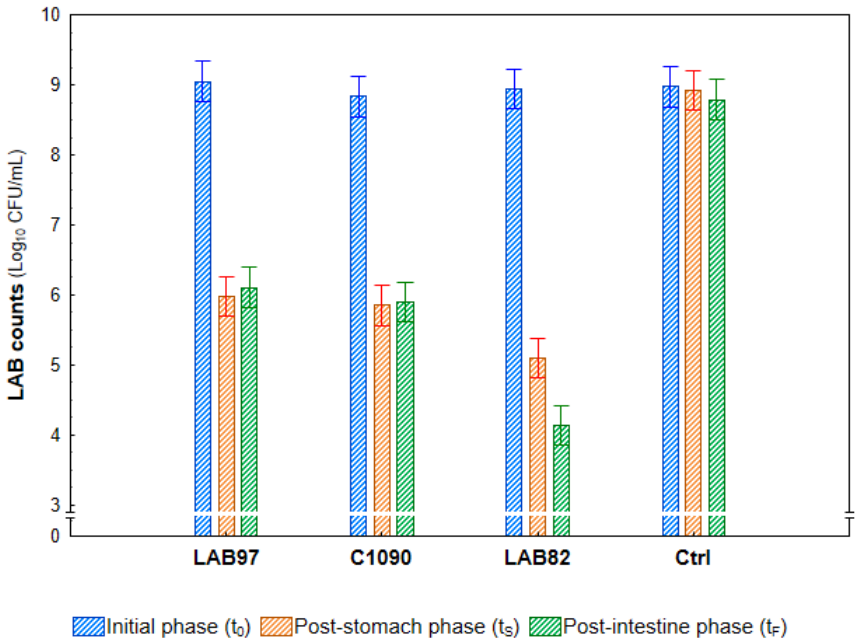

Figure 3.3 - Survival of LAB 97 and C1090 samples and LAB82 and Ctrl as reference and control samples under *in vitro* simulated gastrointestinal conditions. Bars represent the confidence intervals at 95% (Tukey's Test, p=0.05).

In control conditions, all strains (LAB97, C1090 and LAB82) maintained the initial counts (≈ 9 log CFU/ml; p>0.05), demonstrating that the variations observed resulted from the simulation of the digestive conditions. As shown in Figure 3.3, each condition differentially affected the survival of the tested strains. One-hour incubation in stomach simulation (SGF), characterized by acidic pH (2.5), significantly reduced bacterial counts for all the LAB tested (≈3 log reduction for LAB97 and C1090 strains and ≈ 3.5 log reduction for LAB 82).

The following condition (intestinal simulation – SIF) affected the surviving strains differently. LAB97 and C1090 strains maintained viability after two hours of incubation in SIF (counts of about 5.5 Log CFU/ml). On the other hand, a more severe impact on bacterial survival was demonstrated for LAB82 (*Leuconostoc citreum*), which was expected due to its previously assessed sensitivity, showing a further significant decrease of *ca* 1 log CFU/ml. These results show that *L. plantarum* (LAB97) and *W. paramesenteroides* (C1090) had a higher tolerance to bile salt than to acidic conditions, as reported by Sun *et al.*, 2022 and Yadav *et al.*, 2022.

The survival rate was close to 67% for strains LAB97 and C1090 and 46% for LAB82, confirming that LAB82 was the most sensitive strain. As for LAB97 and C1090

reached *ca.* 6 log CFU/mL counts and showed a similar survival capacity, meeting the viability criterion for potential probiotic capacity. (Pereira *et al.*,2021. Garcia *et al.*, 2020. Mir *et al.*, 2018)

3.2. Quality assessment of fermented immature tomato ingredients inoculated with LAB strains as starters

3.2.1. Objective

- Considering that both LAB strains (LAB97 and C1090) as a starter have probiotic potential, we evaluated their performance (as a singular starter) in the fermentation process of immature tomatoes to obtain a healthy and appealing flavour ingredient.

3.2.2. Experimental Design

A batch of ≈ 10 kg of stored immature tomato fruit (-20 °C) was thawed (see 2.1.2). The fruit mixture (belonging to green and intermediate categories in a 1:1 (wt:wt) ratio) was homogenised (see 2.1.2) with the addition of NaCl (1.5 %). The pulp was distributed into jars (1 L), and four sample types (500 ml) were set up in triplicate:

- Pulp fermentation by starters - two types of inoculated samples (Id: LAB97 and C1090);
- Pulp spontaneous fermentation - two types of control samples (Id: CTR-TT sample and CTR-NTT sample).

In the samples to be inoculated (addition of starters) and in one of the control samples (CTR-TT), the pulp was previously thermally treated (110 °C for 2 min) to minimise the influence of the microbiota present in the raw material (Fig. 3.4).

Figure 3.4 - Heat treatment of the pulps (autoclave) before starter inoculation. From left to right, samples at the autoclave and during cooling in water.

After the thermal treatment, only LAB97 and C1090 samples were individually inoculated.

All samples (LAB97, C1090, CTR-TT and CTR-NTT) were put to ferment (T=25 °C; under continuous stirring) for 14 days, and aliquots were taken at regular intervals (0, 24 h, 72h, 7th and 14th days) to assess the following parameters: LAB and Y&M counts, pH, TA, SSC, CIELab, TPC and AOx and organic and phenolic profiles. Sensory analysis was only performed on the last day (14^{th} day) to assess colour, consistency, aroma, flavour, and overall acceptance. Data from the trial were subjected to analysis of variance (ANOVA). Statistically significant differences ($p<0.05$) between samples were determined according to the Tukey test.

3.2.3. Results and discussion

LAB and Y&M counts

The change in the LAB counts in the immature-tomato pulps during the tested period is shown in Fig. 3.5. This gives valuable information on this microbial group's growth capacity to assess the fermentation process's success. As a general trend, LAB counts of all samples showed an increase during the first 72 h, reaching levels that ranged from 7.5 to 9.0 log CFU/ ml. From this date onwards, in both inoculated and control samples, LAB counts were maintained ($p>0.05$) with no further variations until the end of the tested period.

On day 0, the LAB counts of both control samples (<10 CFU/ml and 4 log CFU/ml for CTR_TT and CTR_NTT, respectively) were significantly lower than the inoculated samples, as expected. Interestingly, LAB growth at the end of 72 hours reached similar levels in all samples, regardless of the amount of initial inoculum, with sample LAB97 accounting for the highest value (9 log CFU/ml), achieved earlier at 24 hours. The fact that strain LAB97 was isolated from immature tomato may account for its adaptability to the matrix and its high ability to ferment it. On the one hand, it proved more efficient than strain C1090 showing a higher growth rate in the first 24 hours. On the other hand, in both control samples (spontaneous fermentation), its probable presence can justify the high growth rates of the LAB group observed.

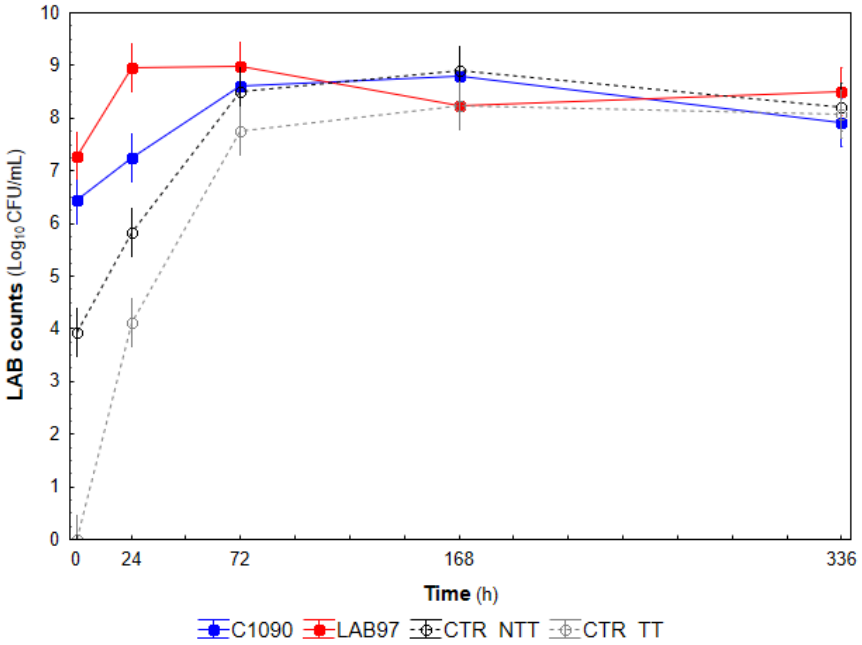

Figure 3.5 - Changes in LAB counts (expressed as $\log_{10}$ CFU/ml) during lactic acid fermentation of immature-tomato pulp samples inoculated with single lactic acid bacteria (LAB) starter cultures (LAB97 and C1090) and in non-inoculated samples (CTR_NTT and CTR_TT) for 14 days. Bars represent the confidence intervals at 95% (Tukey's Test, p=0.05).

These results confirm the growth capacity of both LAB strains in immature tomato pulp with no nutrient supplementation or pH adjustment and the viability of the LAB population (more than > 7 log CFU/ml) up to 2 weeks.

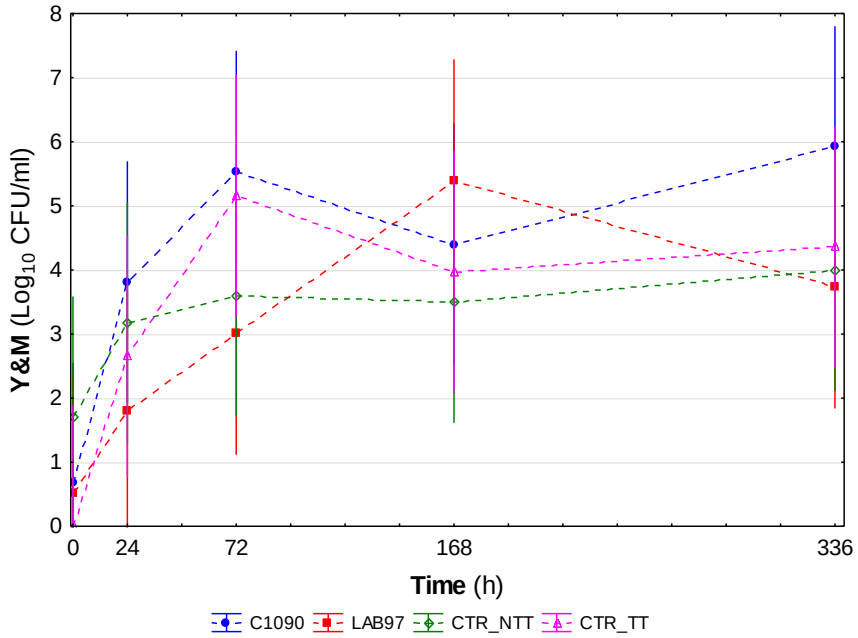

Figure 3.6 - Changes in Y&M counts (expressed as $\log_{10}$ CFU/ml) during lactic acid fermentation of immature-tomato pulp samples inoculated with single lactic acid bacteria (LAB) starter cultures (LAB97 and C1090) and in non-inoculated samples (CTR_NTT and CTR_TT) for 14 days. Bars represent the confidence intervals at 95% (Tukey's Test, p=0.05).

Y&M growth between samples was similar (Fig. 3.6), with no significant differences during the tested period. However, on day 0, sample CTR_NTT had higher counts (2 CFU/ml) than the others (< 10 CFU/ml), which can be justified because it was the only sample not subjected to heat treatment before fermentation. By the end of the tested period, Y&M counts ranged from 3.5 to 6 log CFU/ml ($p>0.05$).

Like the LAB group, yeast growth can also contribute to the release and biotransformation of beneficial chemicals and sugar lowering during tomato fermentation. They can even play an essential role in developing flavours in the resulting fermented products (Cai *et al.*, 2022). According to the similarity of Y&M group growth between the samples, differences in tomato fermentation will be mainly attributed to the LAB group growth.

pH, TA, and SSC changes

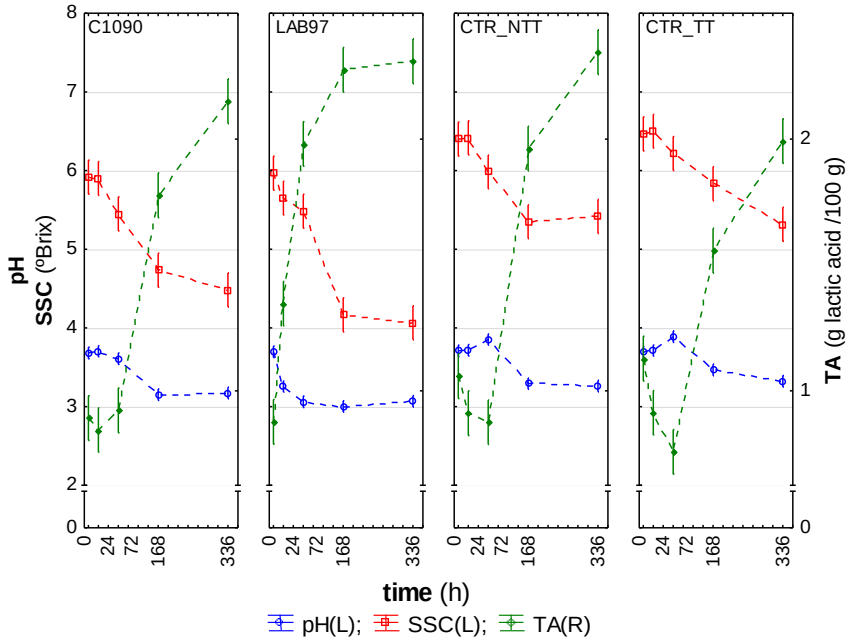

Figure 3.7 - Changes in TA, pH, and SSC during lactic acid fermentation of immature-tomato pulp samples inoculated with single lactic acid bacteria (LAB) starter cultures (LAB97 and C1090) and in non-inoculated samples (CTR_NTT and CTR_TT) for 14 days. Bars represent the confidence intervals at 95% (Tukey's Test, p=0.05).

The pH decrease and the TA increase (inversely correlated) observed in all samples (Fig. 3.7) showed the progressive acidification of the fermented products during the tested period. Simultaneously, the reduction trend of the SSC is related to substrate consumption for microbial growth. These relationships reveal the lactic fermentation occurrence in the immature tomato pulp in all situations.

In sample LAB97, the significant variations of TA occurred earlier (24h) relative to samples C1090 and CTR_TT and CTR_NTT. Similarly, SSC decreased earlier in LAB 97 samples, implying that *L. plantarum* significantly reduced fermentable sugars in vegetables. These characteristics demonstrate this strain's superior efficiency in fermenting immature tomato pulp. These results are consistent with those of Mashitoa *et al.*, 2021 and Di Cagno et al., 2013, who found that the pH of pure mango and carrot fermented with *L. plantarum* rapidly decreased after fermentation.

Organic acid profile

According to the calculated results, only oxalic, citric, lactic, and acetic acids were detectable and quantifiable in the experiment among the organic acid profile (Table 2.2).

Table 3.2 shows the correlations between the TA and the respective acids produced during lactic acid fermentation to determine which is the predominant acid produced during fermentation.

Table 3.2 - Mean values of the titratable acidity (TA) and organic acid contents (oxalic, citric, lactic, and acetic) in the samples (C1090, LAB97, CTR_NTT and CTR_TT) over 14 days.

Sample	Time (h)	TA (g LA/100 g)	Oxalic ac. (mg/100 g)	Citric ac. (mg/100 g)	Lactic acid (mg/100 g)	Acetic acid (mg/100 g)
C1090	0	0.9^{ab}	13.3^{bcd}	945.7^{def}	0^{a}	0^{a}
	24	0.8^{ab}	12.9^{bcd}	886^{cde}	0^{a}	13.6^{abcd}
	72	0.9^{abc}	12.3^{abcd}	711.1^{c}	212.9^{bc}	4.2^{bcde}
	168	1.8^{fg}	14.4^{cde}	125.5^{a}	485.3^{ef}	23.4^{cdef}
	336	2.1^{hi}	12.6^{abcd}	77.3^{a}	$526e^{fg}$	35.8^{defg}
LAB97	0	0.9^{ab}	$12a^{bcd}$	907^{cdef}	0^{a}	0^{a}
	24	1.3^{de}	13.7^{bcde}	981.8^{ef}	258.6^{c}	0^{a}
	72	2.0^{gh}	11.6^{ab}	863.1^{cde}	413.1^{de}	1.8^{ab}
	168	2.3^{i}	16.3^{e}	954.2^{def}	579.5^{fg}	6.7^{abcd}
	336	2.3^{i}	13.1^{bcd}	758.9^{cd}	525.1^{efg}	5.5^{abc}
CTR_NTT	0	1.1^{bc}	12^{abcd}	973.7^{ef}	0^{a}	0^{a}
	24	0.9^{abc}	12.1^{abcd}	1089.7^{f}	0^{a}	5.6^{ab}
	72	0.9^{ab}	12.1^{abcd}	83.2^{a}	194.4^{bc}	10.5^{efg}
	168	2.0^{gh}	10.1^{a}	33.2^{a}	424.4^{de}	7.6^{efg}
	336	2.3^{i}	11.7^{abc}	88.1^{a}	607.9^{g}	13.5^{g}
CTR_TT	0	1.1^{cd}	13.8^{bcde}	934.3^{def}	0^{a}	0^{a}
	24	0.9^{abc}	14.5^{de}	1017.8^{ef}	0^{a}	6.6^{bcde}
	72	0.8^{a}	13.8^{bcde}	498^{b}	122.4^{b}	30.6^{def}
	168	1.6^{ef}	12.6^{abcd}	135^{a}	309.2^{cd}	88.5^{fg}
	336	2.0^{gh}	11.8^{abc}	46.6^{a}	469^{ef}	110.1^{g}

Note: Numbers followed by different letters are statistically different $p \leq 0.05$ (Tukey's test)

The predominant acid in immature tomato pulp (Table 3.2) was citric acid, as expected, since it is the primary organic acid in tomato raw material (Agius *et al.*, 2018), accounting for mean values of 940.2±27.6 mg/100 g (evaluations on day 0).

On the other hand, among the acids quantified, the lactic acid content was the one that best correlated (Table 3.3) with the increase in titratable acidity (TA), attesting to the lactic fermentation that occurred in the samples.

Table 3.3 - Coefficients and statistical significance of the correlation between titratable acidity (TA) and each organic acid.

	R(X,Y)	**R^2**	**P**	**N**
TA *VS* OXALIC ACID	-0.03	0.00	0.848688	60
TA *VS* CITRIC ACID	-0.44	0.19	0.000429	60
TA *VS* LACTIC ACID	0.92	0.85	7.75E-26	60
TA *VS* ACETIC ACID	0.35	0.12	0.006128	60

Note: Red values indicate a significance level of $p<0.05$

Citric acid and oxalic acid showed low correlation coefficients with TA (Table 3.3). Citric acid had an inverse relationship with TA (decreasing levels as time advances). In this sense, the above variations do not seem to depend directly on the fermentation progress (as a metabolite produced by the growth of the microbial population). Regarding oxalic acid (without correlation with TA), no changes ($p>0.05$; Table 3.2) were registered during the tested period, regardless of sample type.

The acetic acid increase (with a low positive correlation with TA, Table 3.3) revealed more significant rises in CTR_TT control samples compared to the other samples. These differences can be attributed to the influence of microbiota diversity in the fermentation process. The acetic acid produced by acetic acid bacteria imparts a sour taste and characteristic aroma. Its presence in fermented products commonly causes an undesirable sour smell and taste (Gomes *et al.*, 2018).

Figure 3.8 shows the lactic acid increments observed over the tested period for all samples, thus confirming, as before, that the fermentation with LAB 97 as a starter was the most efficient.

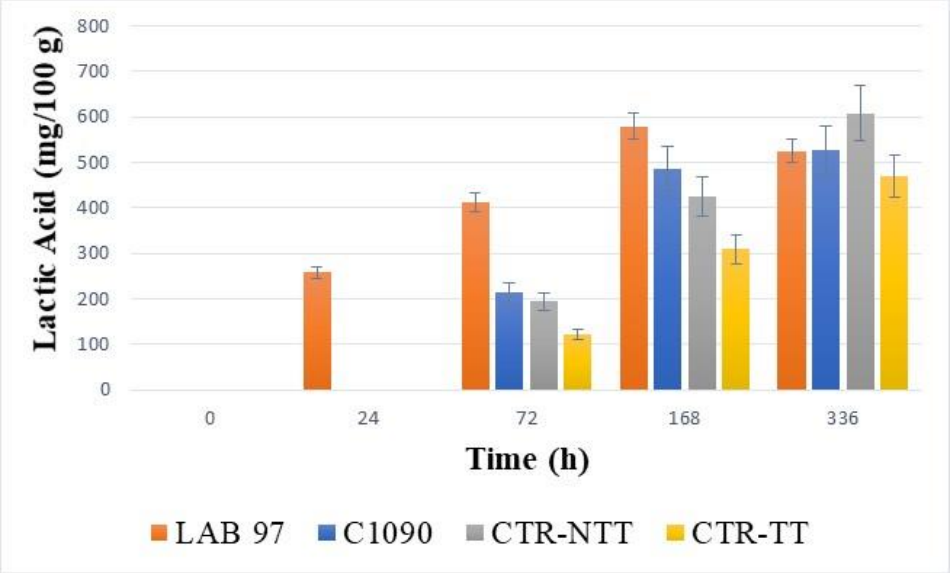

Figure 3.8 - Acid lactic increments (mean±SD) during lactic acid fermentation of immature-tomato pulp samples inoculated with single lactic acid bacteria (LAB) starter cultures (LAB97 and C1090) and in non-inoculated samples (CTR_NTT and CTR_TT) for 14 days.

For the remaining samples (C1090, CTR_NTT and CTR_TT), the lactic acid produced did not show significant differences between them on all the dates assessed.

Bioactive composition (AOx, TPC and phenolic profile)

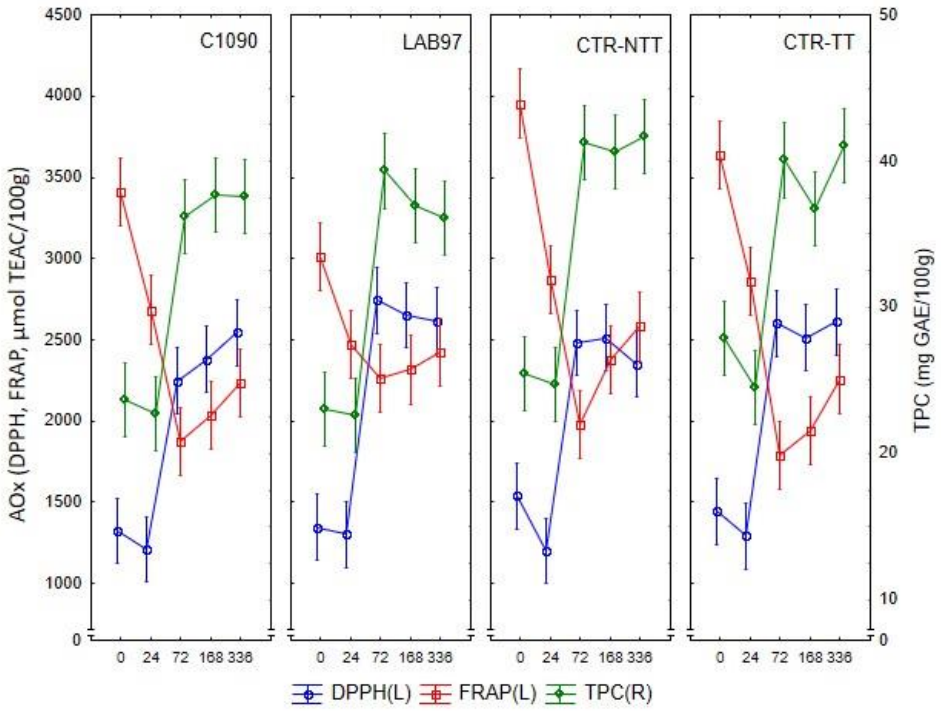

Figure 3.9 - Changes in AOx (DPPH and FRAP) and total phenolic content (TPC) during lactic acid fermentation of immature-tomato pulp samples inoculated with single lactic acid bacteria (LAB) starter cultures (LAB97 and C1090) and in non-inoculated samples (CTR_NTT and CTR_TT) for 14 days. Bars represent the confidence intervals at 95% (Tukey's Test, p=0.05).

Figure 3.9 represents changes in Antioxidant content (DPPH and FRAP method) and Total Phenolic Content (TPC) during 14 days of lactic acid fermentation of immature-tomato pulp samples inoculated with single lactic acid bacteria (LAB) starter cultures (LAB 97 and C1090), and non-inoculated samples (CTR-NTT and CTR-TT).

Changes in all samples' antioxidant capacity by DPPH method and total phenolic content (high positive correlation, r = 0.93, Table 3.4) over time show a similar pattern. There is a significant increase ($p<0.05$) between the 24 and 72-hour periods (values nearly doubled). After this increase, the values were maintained until the evaluation period's end. The high correlation suggests that the ferments' antioxidant capacity is attributed to the predominant phenolic compound composition. Furthermore, the significant variation in AOx and TPC matches the significant growth phase of the LAB population. Other authors have mentioned that it is possible to release antioxidant compounds during fermentation which might justify this behaviour. The antioxidant capacity tested by the FRAP method is inversely correlated with TPC, indicating that compounds other than phenolics with antioxidant capacity could probably be present.

Table 3.4 - Coefficients and statistical significance of the correlation between AOx (FRAP and DPPH methods) and total phenolic content (TPC) of all samples over 14 days.

	TPC	DPPH	FRAP
TPC	1.00 (p = --)	0.93* (p = 0.00)	-0.66* (p = 0.00)
DPPH		1.00 (p = --)	-0.67* (p = 0.00)
FRAP			1.00 (p = --)

*The correlation was significant

The HPLC-DAD method for detecting and quantifying phenolic compounds was not validated for the sample concentrations tested. The phenolic concentration in the sample was very diluted, concerning the limits of quantification used (calibration curves). Therefore, it was only possible to identify the phenolic compounds present in the samples for the different test dates and the results are presented in Table 3.5. The compounds that were not detected in the experiment were kaempferol, naringenin, and quercetin.

Table 3.5 – Phenolic compounds identified on the samples (LAB97, C1090, CTR_NTT and CTR_TT) during the tested period (red means that the phenolic compound was not found in the sample, and blue means that it was identified but not quantified).

Sample	Time (h)	Catechin	Chlorogenic	Hydroxybenzoic	Vanillic Acid	Caffeic	Syringic	Coumaric	Rutin	Ferulic	Naringin
C 1090	0	ND	ND	NQ	ND	ND	ND	NQ	ND	NQ	ND
LAB 97	0	ND	ND	NQ	ND	ND	ND	NQ	ND	NQ	ND
CTR-NTT	0	ND	ND	NQ	ND	ND	ND	NQ	ND	NQ	ND
CTR-TT	0	ND	ND	NQ	ND	ND	ND	NQ	ND	NQ	ND
C 1090	24	ND	ND	NQ	ND	ND	NQ	NQ	ND	ND	ND
LAB 97	24	ND	ND	NQ	ND	ND	NQ	NQ	ND	ND	ND
CTR-NTT	24	ND	ND	NQ	ND	ND	ND	NQ	ND	ND	ND
CTR-TT	24	ND	ND	NQ	ND	ND	NQ	NQ	ND	ND	ND
C 1090	72	ND	ND	NQ	ND	ND	NQ	NQ	ND	ND	ND
LAB 97	72	ND	ND	NQ	ND	ND	NQ	NQ	ND	ND	ND
CTR-NTT	72	ND	ND	NQ	ND	ND	NQ	NQ	ND	ND	ND
CTR-TT	72	ND	ND	NQ	ND	ND	NQ	NQ	ND	ND	ND
C 1090	168	ND	ND	NQ	NQ	ND	ND	NQ	ND	NQ	ND
LAB 97	168	ND	ND	NQ	NQ	ND	NQ	NQ	ND	NQ	ND
CTR-NTT	168	ND	ND	NQ	ND	ND	NQ	NQ	ND	NQ	ND
CTR-TT	168	ND	ND	NQ	NQ	ND	NQ	NQ	ND	NQ	ND
C 1090	336	ND	ND	NQ	NQ	NQ	NQ	NQ	ND	NQ	ND
LAB 97	336	ND	ND	NQ	ND	NQ	NQ	NQ	ND	NQ	ND
CTR-NTT	336	ND	ND	NQ	ND	ND	ND	NQ	ND	NQ	ND
CTR-TT	336	ND	ND	NQ	ND	ND	ND	NQ	ND	NQ	ND

Figure 3.10 displays chromatograms for each sample on 7-day at wavelengths 280 and 325 nm. Day 7 was selected because it corresponded with the post-fermentation period when an increase in total phenolic content was observed. The chromatograms do not show many differences between samples and do not add information to table 3.5.

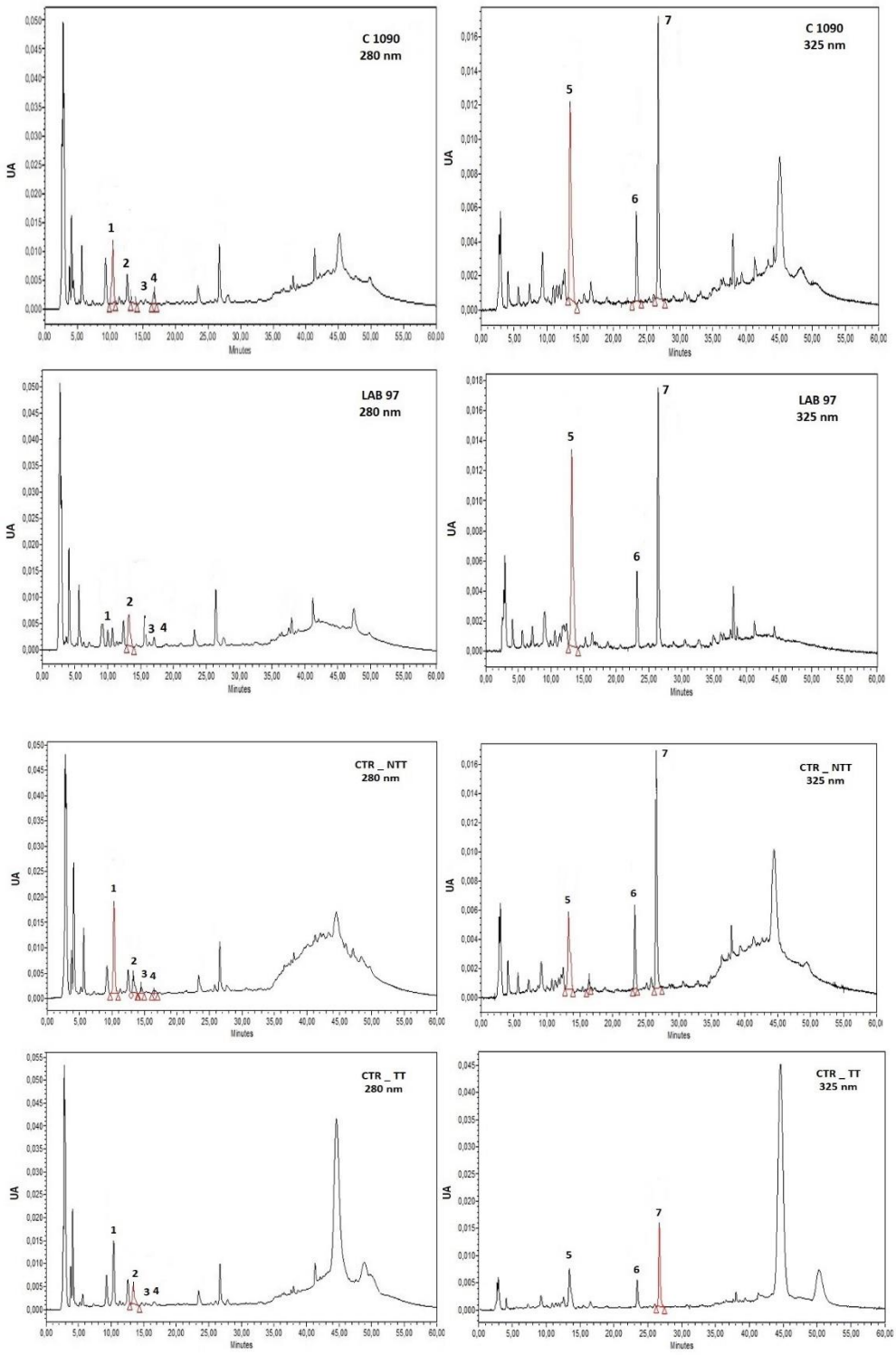

Figure 3.10 - HPLC-DAD chromatograms for the optimized condition at λ = 280 & 325 nm on day 7 of C1090, LAB 97, CTR _NTT and CTR _ TT samples. **(**Peak identification: 1 - catechin; 2 - hydroxybenzoic acid; 3 - vanillic acid; 4 - syringic acid; 5 - chlorogenic acid; 6 - cumaric acid; 7 - ferulic acid. The red values are the mean amount of phenolic compound in the sample (mg/100g)

CIELab colour

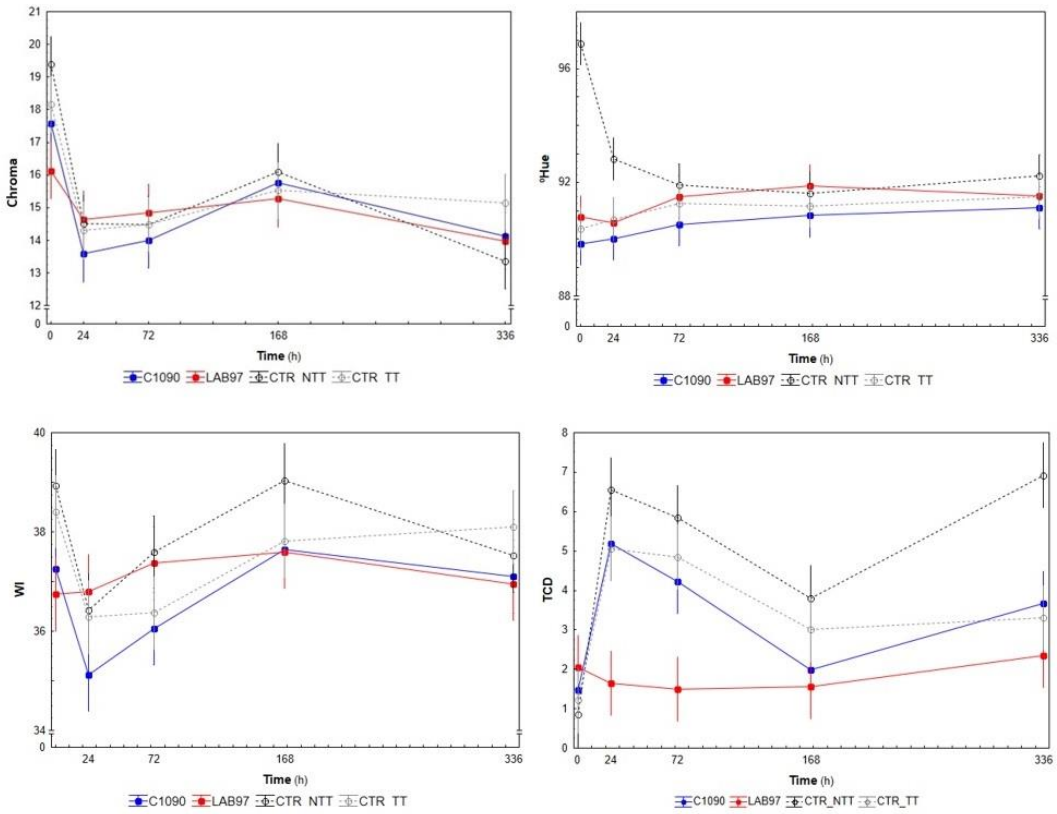

Figure 3.11 - Average values of the instrumental colour parameters a) Chroma (C*), b) Hue angle (°h), c) whiteness index (WI) and d) Total Colour Differences (TCD) of immature-tomato pulp samples inoculated with single lactic acid bacteria (LAB) starter cultures (LAB 97 and C1090) and non-inoculated samples (CTR_TT, CTR_NTT) over 14 days. Bars represent the confidence intervals at 95% (Tukey's Test, p=0.05).

During fermentation, the chroma of the samples follows a similar pattern (Figure 3.10, a), a significant decrease in the first tested interval (24 h) and relative maintenance in the subsequent period. LAB 97 was an exception to this pattern, as it maintained the chroma throughout the testing period ($p>0.05$), indicating higher levels of colour purity.

The sample CTR_NTT had significantly higher Hue values than the others at the end of 24 hours. These Hue values indicate a greener shade in this sample, which could be explained by the fact that it is the only one that has not been heat-treated. However, after 72 hours, the tonality of all samples converged to similar values. (Figure 3.10, b).

The darkening of the ferments, as measured by a decrease in WI values, followed a consistent pattern among all samples (Figure 3.10, c). Significant changes ($p<0.05$) occurred during the first 24 hours, followed by relative maintenance. The LAB97 sample

was again the exception because it did not show significant WI changes over the test period.

The TCD values (Figure 3.10, d), which express colour changes relative to the initial condition, revealed significant differences between samples during the fermentation phase (72 hours). During this period, there was a clear differentiation between LAB 97 (1.5, not very distinct differences) and the others (>4, very distinct differences) according to table 2.1 criteria (Allegretti *et al.*,2009). WI was the most correlated parameter with the TCD, indicating that the visible colour changes were expressed by pulp darkening.

Sensory analysis

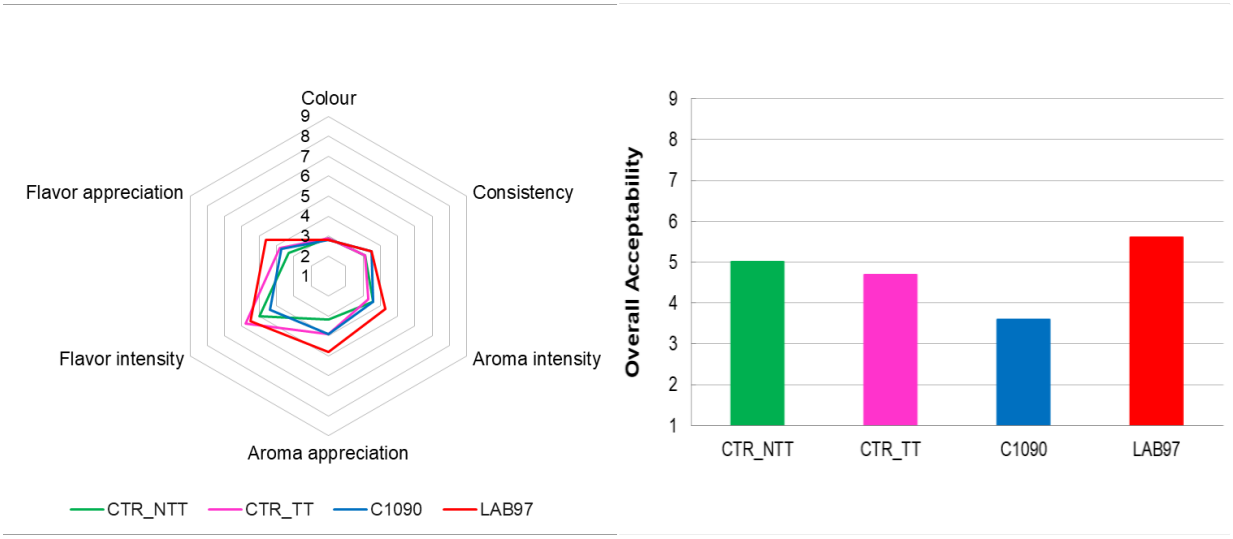

Figure 3.12 – Average scores for sensory Attributes (colour, aroma, flavour, aroma, consistency) of the samples (LAB 97, C1090, CTR_TT, CTR_NTT) on the 14th day (on the left) and the mean scores for the samples overall acceptance (on the right).

The sensory evaluation of the fermented products (figure 3.12) was performed only on the last day of fermentation by panellists who distinguished the sensory attributes, colour, aroma appreciation, flavour appreciation, aroma intensity, consistency, and overall acceptability of the samples.

In Figure 3.12, the colour and consistency mean scores of the inoculated and non-inoculated fermented samples were similar ($p>0.05$). As a result, it is unlikely that the samples' colour and texture significantly impact the sensory acceptance of this product.

It is well-known that the ability of aroma development might change depending on the strain and the metabolic diversity of the several lactic acid fermentation strains (Fonseca et al., 2021). In this study, the sample inoculated with LAB97 had a more pungent smell than the other samples. Aroma appreciation for CTR-TT, CTR-NTT , and C1090 samples was similar, while LAB97 was the most appreciated sample on the aromatic feature.

Immature tomatoes fermented with LAB97 obtained the best sensory acceptance using *Lactiplantibacillus plantarum* strain. Therefore, this strain has been widely used as a starter culture in various fruit and vegetable fermentation processes, contributing favourably to organoleptic properties, flavour, and texture (Todorov and Franco, 2010).

3.3. Interim findings

Although spontaneous fermentation of immature tomato pulp has been possible (both CTR samples), this option makes product quality neither predictable nor controllable. Inoculated fermentation using pure cultures as the starter is advised for optimisation and quality control of the ingredient. LAB 97 was selected as it is the most efficient LAB to ferment the product.

In addition, the immature tomato pulp inoculated with strain LAB 97 maintained microbial counts above 6 log CFU/ml until the end of the tested period. This behaviour can be considered promising for the probiotic potential of the ingredient, given that fermented products claim a minimum of 10^6 viable probiotic bacteria per gram of product at the time of purchase, according to international standards (Kun, 2008).

4. DEVELOPMENT OF HEALTHY SAUCE FORMULATION BASED ON FERMENTED INGREDIENT

4.1. Selection of ingredients and setup of technological operations

4.1.1. Objective

- The present trial aimed to formulate a sensory appealing, nutritious, and probiotic potential sauce based on blending fermented green tomatoes with other valuable ingredients. The experiment involved ingredient selection and formulation (STEP 1) and the development of the technology sequence (STEP 2).

4.1.2. Experimental Design

STEP 1 - A minimum of 65% fermented tomato (base ingredient) was considered in the sauce formulation to ensure its potential probiotic nature. Additional ingredients were trialled to design the sauce (secondary ingredients); their proportions and justification beyond nutritional and bioactive properties are presented in Table 4.1

Table 4.1- Ingredients tested in the sauce formulation

Ingredients	**%**	**Function(s) in sauce**
Immature tomato ferment	65	Probiotic potential
Avocado	20	Emulsifier, colourant, and flavour enhancer properties
Honey	10	Sweetener and mitigation of the perceived sharp acidity in tomato ferments
Option 1: Coriander or Option 2: Parsley	4	Flavouring and colour enhancers

Green tomato ferment (*cv*. H1015) was prepared by inoculating LAB97 (10^8 CFU/ml) (as described in 2.2.1, Material and Methods part). After 72 h, the ferment LAB counts maintained > 8 log CFU/ml, and it was used in the sauce formulation. Raw

materials were used to test the two formulas, given the proportions described in (Table 4.1). For this purpose, the secondary ingredients (honey, avocado, and parsley (Id: Pa) or coriander (Id: Co)) were previously homogenized (Thermomix, maximum speed, 1 min) and then mixed with the tomato fermented. The mixture was performed by a short homogenization (Thermomix, maximum speed, 15 seconds). The pulps were stored in glass jars and evaluated for the following parameters: pH, SSC, and CIELab at regular intervals (0h, 24h, 72h and 168 hours), and sensory analysis was performed immediately after pulps preparation.

STEP 2 - In the second step, the aim was to evaluate two technological strategies to reduce the level of contamination of secondary ingredients (avocado and parsley) and guarantee high stability for the sauce. For this purpose, three sauce samples were prepared as follows:

- The partial decontamination sample (**Id: PD**): Decontamination of the avocado was carried out by a heat shock treatment by immersing the whole fruit in a boiling water bath for 30 s, followed by cooling (ice bath), drying (absorbent paper), peeling, and stone removal. The same procedure was followed for parsley but reduced treatment time (15 s) due to the plant's delicate tissues. Then, the secondary ingredients were weight and mixed in a ratio of 4:2:1 (avocado, honey, and parsley).
- The sterilized samples **(Id: St**): The mixture of ingredients (avocado, honey, and parsley, in the ratio of 4:2:1) were sterilized in an autoclave for 2 min at 110 °C and cooled down in an ice bath.
- The control samples (**Id: F**): The samples were prepared by mixing the secondary ingredients (avocado, honey and parsley in a ratio of 4:2:1) blended raw without further treatment.

To assess the decontamination level, all samples, heated and not heated, were immediately evaluated for TAPC (total aerobic plate counts) and Y&M (yeasts and moulds) counts.

4.1.3. Results and discussion

pH and SSC changes (STEP 1)

Table 4.2 shows the pH and SSC changes of the first step in sauce production. The use of coriander or parsley in the formulations did not affect the pH and SSC values, which were within the range of 3.3 – 3.4 and *ca*. 16 -17 ºBrix, respectively, throughout the tested period. The low pH values represent a benefit for the formulations' stability.

Table 4.2 - pH and SSC changes (mean) of the sauces **(**7 days**)**.

Sample	Time (h)	pH	SSC
Sauce with Coriander	0	3.3^{c}	17.1^{b}
	24	3.3^{c}	16.7^{a}
	72	3.4^{b}	16.7^{a}
	168	3.4^{a}	16.7^{a}
Sauce with Parsley	0	3.4^{a}	16.9^{ab}
	24	3.4^{ab}	16.9^{ab}
	72	3.4^{b}	17.0^{b}
	168	3.4^{a}	15.9^{c}

Note: Mean followed by different letters are statistically different (P<0.05; Tukey's test)

CIELab colour (STEP 1)

Table 4.3 - CIELab Colour evaluation (mean) of the sauces (7 days).

Sample	Time (h)	Hue	WI	TCD
Sauce with Coriander	0	111.2^{d}	44.7^{ab}	1.1^{c}
	24	106.8^{a}	44.8^{ab}	5.1^{ab}
	72	105.4^{ab}	44.0^{ab}	4.6^{ab}
	168	102.9^{c}	45.2^{ab}	5.6^{ab}
Sauce with Parsley	0	112.2^{d}	46.4^{a}	0.5^{c}
	24	106.7^{a}	45.8^{ab}	3.0^{d}
	72	106.0^{ab}	44.0^{ab}	4.2^{cd}
	168	104.2^{bc}	42.9^{b}	5.7^{b}

Note: Mean followed by different letters are statistically different (P<0.05; Tukey's test)

Table 4.3 shows the evaluation of the CIELab colour parameters of the pulps (coriander and parsley) for seven days under refrigeration conditions. Both pulps showed similarities (Hue, WI) on day 0. However, the colour changes expressed by increasing TCD (< 6 for both pulps at the end of 7 days) reflect remarkable colour differences (table 2.1). The colour changes in both samples, with no differences, are attributed to significant

changes in pulp hue (°h) and browning level (WI), which may be related to enzymatic oxidation reactions occurring during storage.

Sensory evaluation (STEP 1)

The informal tasting of the formulations (parsley or coriander addition) obtained a favourable global appreciation in terms of taste, colour and consistency. As none of them stood out, we decided to continue the development of the sauce prototype using parsley, as it is considered an ingredient of high bioactive and medical interest. (Farzaei *et al*., 2013)

Total aerobic Plate (TAPC) and Y&M counts (STEP 2)

Table 4.4 - Mean of microbial counts (Log CFU/ml) after heat treatments (PD, St samples) and the raw contamination of the mixed secondary ingredients (Sample F).

	TAPC (log CFU/mL)	**Y&M** (log CFU/ml)
F	6.4^{c}	1.9^{b}
PD	1.3^{a}	0.0^{a*}
St	3.4^{b}	0.0^{a*}

Note: Mean followed by different letters are statistically different ($P<0.05$; Tukey's test); *< 10 CFU/mL

The two treatments tested effectively eliminated the initial contamination of the blended ingredients (Table 4.4). For the TAPC group, reductions of about 3 log cycles were recorded. After treatments, the Y&M group's counts were less than 10 CFU/ml. However, the sample size was small (≈ 200 g), and the decontamination efficiency will need to be tested when scaling up the work.

4.2. Quality attributes of the sauce stored under refrigeration

4.2.1. Objective

- To evaluate the storage stability of prototype sauces according to two technological options during refrigerated storage (21 days - 5°C).

4.2.2. Experimental Design

As mentioned above, three samples were prepared in triplicate to assess the sauce's quality and refrigeration storage capacity. The samples (PD, St and F) were kept in glass jars at 5 °C for 21 days. Aliquots were taken at regular intervals (0, 7, 14, and 21 days) for the following analysis: LAB, Y&M and TAPC counts (CFU/ml), pH, solid soluble content (SSC, °Brix), CIELab parameters (Chroma, °h, WI, TCD), total phenolic content (TPC, Folin-Ciocalteu method, mg GAE/100 g FW), and antioxidant activity (AOx, DPPH method, µmol TEAC/100 g FW) and sensory analysis (9-point hedonic scale, 13 panellists).

4.2.3. Results and discussion

LAB and Y&M counts

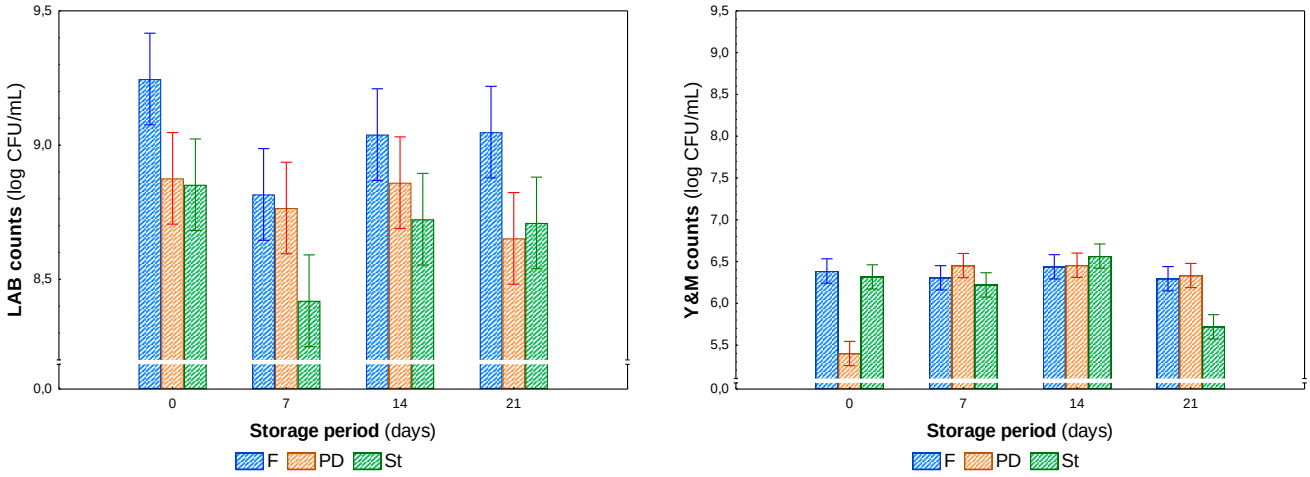

Figure 4.1 – (a) LAB and (b) Y&M count changes (expressed as $\log_{10}$ CFU/ml) for the sauce samples (F, PD and St) during refrigerated storage (21 days; 5 °C). Bars represent the confidence intervals at 95% (Tukey's Test, p=0.05).

The microbiological counts for all samples during storage reveal the relative dominance of LAB (ranging from 8.4 to 9.2 log CFU/ml) vs Y&M content (ranging from 6.0 to 6.5 log CFU/ml) (Fig. 4.1) (except for PD sample at day 0).

Samples that underwent heat treatment (PD and St) consistently had lower LAB counts than the fresh sample (F) for each examined date (Fig. 4.1, a) since it is the only sample that did not undergo decontamination treatment. However, this tendency was only significant ($p<0.05$) on day 0. From that date onwards, the differences between samples never exceeded one log cycle. Concerning the Y&M group (Fig. 4.1, b), the counts were similar ($p>0.05$) between samples and throughout the tested period.

The Total Aerobic Microbial Counts (TAPC, not shown) and LAB counts were similar ($p>0.05$), indicating that lactic acid bacteria in the sauce samples play a fundamental part in the microbial population. On the other hand, adding 65% of the ingredient fermented tomato to the sauce formulation ensures a sufficient amount of lactic acid bacteria in the finished sauce.

LAB and Lactobacillus levels were reported for olives produced in Italy, Greece, Portugal, Spain, and the United States, in the order of 10^4 to 10^8 cfu/g and were between 30 and 200 days (Rezac *et al.*, 2018).

When the dominant microorganism levels of lactic bacteria flora are $>10^8$ cfu/g, the fermented products are considered "unsatisfactory" (Health Protection Agency, 2009). Lactic acid bacteria can grow well at refrigeration temperatures. However, microbiological levels remained stable over the 21 days (5 °C) for all the prototype sauces tested regardless of the treatments applied to the ingredients.

The assessed pH value up to the end of storage for all prototypes, below 4.0, is considered a threshold for product stability by restricting the microbiota to acid-tolerant microorganisms (Kumar and Barman, 2016). In addition to the low pH value of this sauce, the microbiological safety aspects can be further justified by the content of organic acids and natural antimicrobials present, preventing the growth of most pathogenic bacteria (Ribeiro *et al.*, 2018).

As mentioned by (Centre for food safety, 2014), spoilage will eventually occur at about 10^9 cfu/g due to lactic acid production. If Gram-negative bacteria predominate (such as Pseudomonas), spoiling is likely apparent at 10^7 to 10^8 cfu/g, as evidenced by the onset of staining, discolouration, and slime. However, no significant changes were shown in the pH and sensory quality parameters measured for all the samples over the studied period (see sections below).

It should be noted that whereas the microbiological standards refer to values of CFU/g of product, the data on this investigation study expressed CFU/ml. The sauce has a density >1, meaning our results could be overestimated. Nevertheless, the values estimated in our sauce´s prototypes are close to the limits admitted in these products. Other decontamination treatments should be investigated to guarantee the required conformity. Nevertheless, our sauce prototypes' estimated LAB counts are near the

permitted limits for these products. Other decontamination treatments should be investigated to guarantee the required conformity.

pH and SSC changes

The samples' pH and SSC assessment during storage (21 days) are shown in Table 4.5. For each type of sample (F, PD and St), the SSC changes were not significant throughout storage (21 days), with one exception (sample PD on day 7) (Table 4.5), reflecting maximum variations in the order of 1ºBrix. Comparing SSC values between samples highlights sample PD with higher values (around 14 ºBrix). This difference is not justified by the treatments applied to the secondary ingredients. It might be attributed to the inherent variability of the raw materials used in their preparation.

The pH variations of the samples showed no consistent trend, neither between sample types nor during refrigerated storage. The variation range of 3.5 ±0.1 showed that the sauce's acidity was stable for all the formulations tested, regardless of the significance marked (Table 4.5).

Table 4.5 - SSC and pH mean values of the samples (F; PD and St) during the storage period (21 days, 5 °C)

Id_sample	***time (days)***	***SSC (°Brix)***	***pH***
F	0	12.6^{a}	3.4^{b}
	7	12.8^{ab}	3.5^{ad}
	14	12.6^{a}	3.4^{ce}
	21	12.2^{a}	3.5^{a}
PD	0	14.3^{ef}	3.4^{b}
	7	14.9^{f}	3.6^{d}
	14	13.9^{de}	3.4^{ce}
	21	13.9^{de}	3.5^{a}
St	0	13.6^{bde}	3.4^{be}
	7	12.8^{ab}	3.5^{ad}
	14	12.5^{a}	3.4^{ce}
	21	13.0^{abd}	3.5^{a}

Bioactive composition (TPC, AOx)

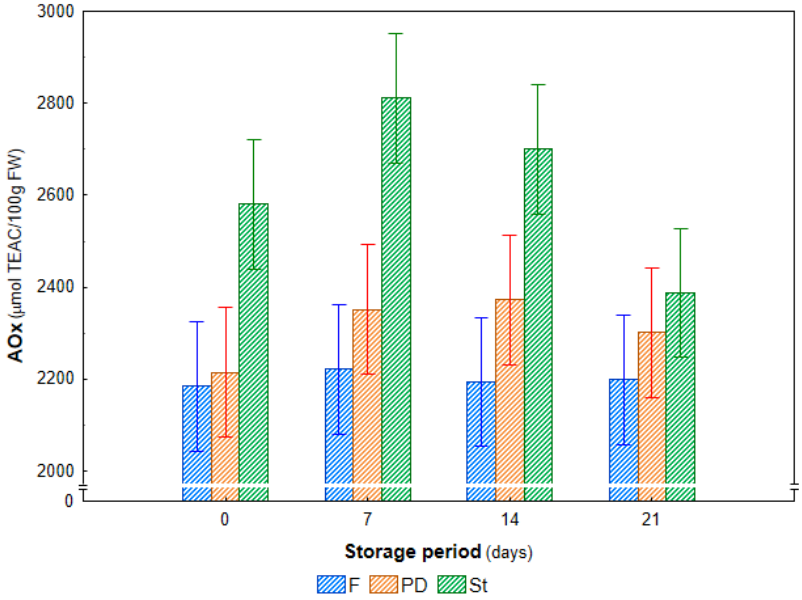

Figure 4.2 - Antioxidant Capacity (AOx, DPPH method) changes (expressed as µmol TEAC/100 g) for the sauce samples (F, PD and St) during refrigerated storage (21 days; 5 °C). Bars represent the confidence intervals at 95% (Tukey's Test, p=0.05).

The antioxidant capacity of each sample (Fig. 4.2), ranging from 2200 to 2800 µmol TEAC/100 g, remained stable throughout the tested period. Nevertheless, differences were detected between sample types. The St sample accounted for higher values ($p<0.05$) than others (F and PD samples) until day 14. Hydrolysis of some compounds due to heat treatment could explain the increased antioxidant activity on St samples. Previous studies have reported that non-enzymatic reaction products might have formed during heat treatment, resulting in increased antioxidant activity (Nicoli *et al.*, 1999; Choi *et al.*, 2006). These results are consistent with Dewanto *et al., 2002,* Turkmen *et al*., 2005, and Francisco and Resurreccion (2009), as the authors reported that heat treatments enhanced antioxidant capacity in pepper, green bean, broccoli, spinach, sweet corn, and peanut.

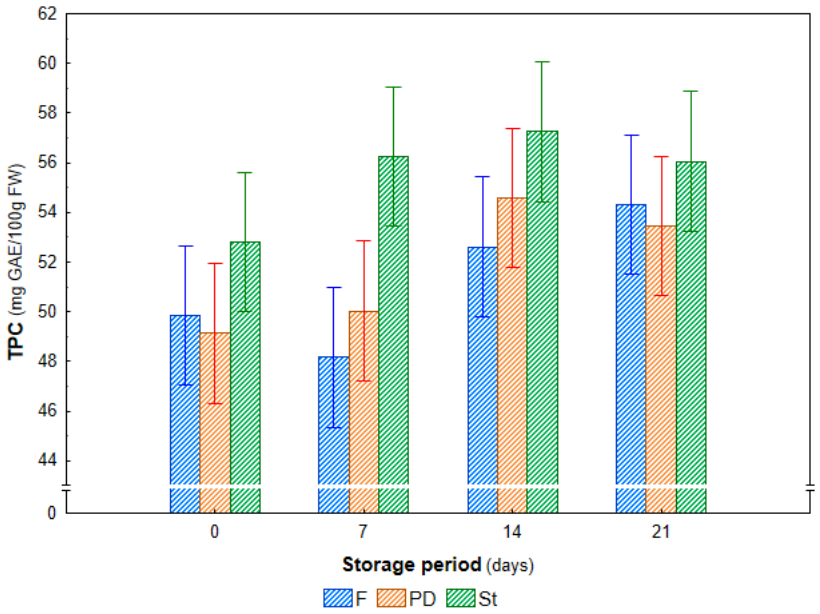

Figure 4.3 - Total Phenolic Content (TPC) changes (expressed as μmol TEAC/100 g) for the sauce samples (F, PD and St) during refrigerated storage (21 days; 5 °C). Bars represent the confidence intervals at 95% (Tukey's Test, p=0.05).

The TPC results in Fig. 4.3 indicate non-significant variations among samples within 48 to 57 mol TEAC/100 g. Although the St samples have accounted for higher ($p>0.05$) mean values than the others, it was confirmed the TPC maintenance during the whole storage period.

The levels of phenolic compounds pointed out for all samples at the end of the storage period can be categorised as low phenolic compound products because they have contents lower than 500 mg GAE/100 g (Paz *et al.*, 2015).

In the storage period, the samples' chroma (Fig 4.4, a) follow a similar pattern, with a significant increase in the first seven days and a relative decrease in the following period. The Fresh sample was an exception, with the chroma remaining stable throughout the storage time ($p>0.05$), indicating higher levels of colour purity.

During storage, the Fresh sample (F) maintained significantly higher Hue values (Fig 4.4 b). These hue values indicate a greener shade in this sample, which could be because it is the only sample that has not been thermally treated. On the other hand, the Partial decontaminated sample (PD) had the lowest hue value, which could be justified given the thermal treatment.

CIELab colour

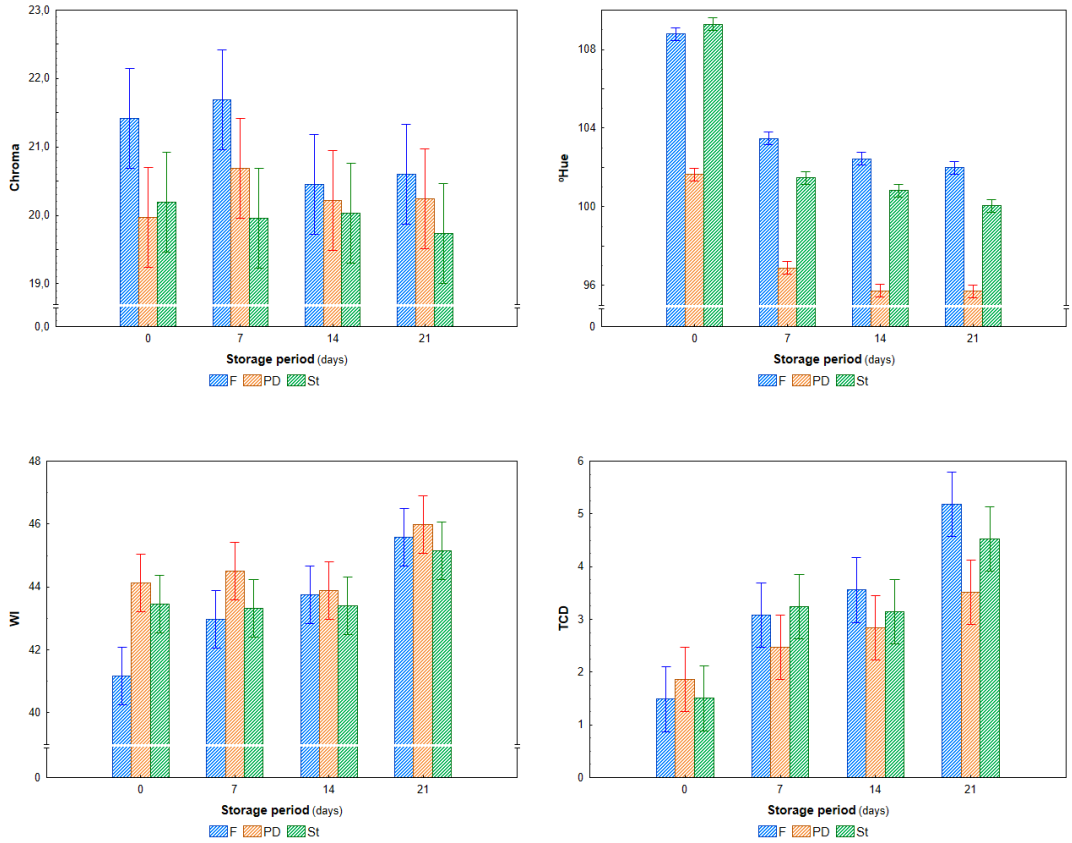

Figure 4.4 - Average values of the instrumental colour parameters a) Chroma (C*), b) Hue angle (°h), c) whiteness index (WI), and d) Total Colour Differences (TCD) of sauce samples (F, PD and St) during refrigerated storage (21 days; 5 °C). Bars represent the confidence intervals at 95% (Tukey's Test, p=0.05).

The WI values of all samples (Fig 4.4, c) increased significantly during storage, with a significant increase on the last day of storage. This increase in sample WI values represents a whitening trend in the samples. TCD values have been shown in Figure 4.4 d, showing the sharp increase trend in sauce samples.

Sensory analysis

Figure 4.5 shows the ratings assigned to the sensory attributes (colour, consistency, aroma and taste) and Global Appreciation evaluated in the samples (F, PD and St) throughout the storage (21 days, 5 °C). The sensory panel's evaluation of the samples' colour attribute did not show significant variations along the storage for each sample type (Fig. 4.5 a).

However, the scores assigned to the PD sample were consistently lower than the others, obtaining on day 0 values below the scale's midpoint (Fig. 4.5, b). It shows that partial decontamination of the ingredients was the treatment that most influenced the colour of the sauce. These results agree with the significant variations evaluated in the CIELab parameters that showed high browning levels (highest WI) and lower greenishness (lowest °h) in these samples. (Fig 4.4)

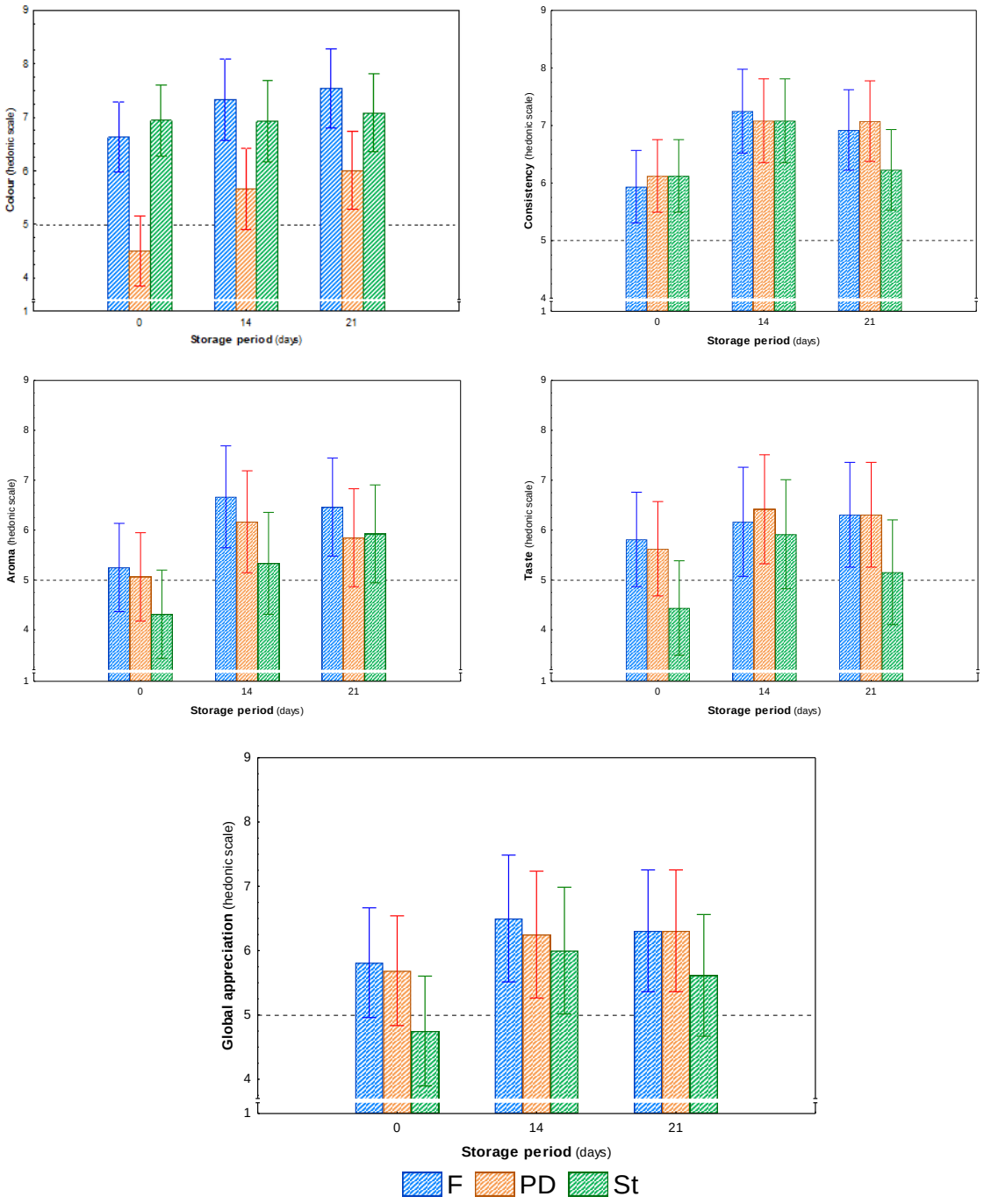

Figure 4.5 - Average sensory scores for the attributes (a: colour, b: consistency, c: aroma, d: taste, and e: global appreciation) of sauce samples (F, PD and St) during refrigerated storage (21 days; 5 °C). Bars represent the confidence intervals at 95% (Tukey's Test, p=0.05).

All samples were rated similar in terms of the consistency attribute ($p>0.05$) and above the scale's midpoint during the storage period. Thus, the decontamination treatments did not influence the sauce's consistency or the samples' overall acceptability. Despite the non-statistical significance, the mean ratings of taste, aroma and overall acceptance of the samples express the same relative variations on the three dates evaluated. They reveal that sterilisation treatment negatively affected the taste and aroma of the sauce (St) right after preparation (day 0). In the remaining storage, all the samples obtained sensory acceptance, with the St samples with ratings close to the rejection level.

These results concluded that the formulated sauces presented overall acceptance levels above the acceptance threshold, nutritional and functional value (based on the blended ingredients), and probiotic potential. The tested decontaminated treatments did not improve the sauce stability during the refrigerated storage (5 °C, 21 days).

4.3. Interim findings

The prototype sauce (65% of GT ferments with 35% blended ingredients: avocado, honey, and parsley, in a 4:2:1 ratio) proved viable after 21 days of storage (5 °C) with sensory appreciated identity and valuable quality characteristics. The prototypes showed functional characteristics with antioxidant activity, namely by the presence of phenolic compounds. The microbiological analyses indicated the stability of the lactic bacteria population (21 days, 5 °C), but with LAB counts close to the limits allowed for similar products. These results highlight the need for further research and development of the sauce´s prototypes being considered, namely to look into alternative decontamination methods for the ingredients to be mixed with the fermented tomato ingredient.

5. GENERAL CONCLUSIONS AND FUTURE WORK

5.1. General conclusions

From this work, we can conclude the following:

- The *in vitro* gastrointestinal digestion simulation results showed that LAB97 and C1090 counts reached *ca.* 6 log CFU/ml, meeting the viability criterion for potential probiotic capacity.
- To obtain a valuable ingredient based on lactic acid fermentation of immature tomato fruit (*cv.* H1015), it proved necessary to perform controlled fermentation using pure cultures as initial inoculum (starter) to speed up the fermentation process and control ingredient characteristics. LAB 97 was selected as it is the most efficient LAB to ferment the GT.
    - The GT ferments using LAB97 as a starter maintained microbial counts above 7 log CFU/ml until 14 days. This behaviour can be considered promising for the probiotic potential of this ingredient.
    - The GT ferments using LAB97 as a stater is an exciting ingredient. It offered probiotic potential, pertinent bioactive composition (phenolic compounds), and sensory acceptability (taste and aroma). However, direct intake is not advisable due to its extreme acidity (*ca.* pH 3). Furthermore, using it in a mixture with other components is advisable to develop sauce formulations instead.
- The prototype sauce (65% of GT ferments with 35% blended ingredients: avocado, honey, and parsley, in a 4:2:1 ratio) proved to be a viable sauce after 21 days of refrigerated storage (5 °C).
    - The product was maintained until the end of the storage with good sensory quality attributes and valuable antioxidant composition, meeting the microbiological criteria for this product, regardless of the treatments performed on the secondary ingredients.

5.2. Future work

Some interesting topics that could be developed as a follow-up to the strategy for valorising this GT fruit are highlighted.

- **Concerning the fermented GT as an ingredient**: A more detailed compositional analysis should be undertaken. In the phenolic profile characterisation of the samples, it was observed that the sample concentration did not allow an adequate quantification of the identified phenolic compounds. This characterisation needs to include a previous concentration step of the samples.
- **Concerning the prototype sauce developed**: More detailed compositional characterisation and probiotic capacity assessment are needed as a final food product. Assessment of alternative preservation treatments besides heat, such as High Hydrostatic Pressure (HPP), to achieve sauce´s stabilisation for extended shelf life periods.

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