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Master's degree in
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Impact of different disruptive pre-treatments on *Lactiplantibacillus plantarum* growth and antioxidant properties in broccoli stalks (*Brassica oleracea* var. italica)

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Abstract

Food waste is one of the most important and relevant problems concerning the whole agricultural and food industry. Its impact not only affects the food security of a big part of the world, but also the economy and several social aspects of the developed countries. All this without considering the influence that food waste impose on climate change. Vegetables represent a huge part of the amount of food that annually gets wasted, due to plants diseases during cultivation, harvesting, storage and distribution, to non-utilization of the products, incorrect handling during all phases, including the households and discarding of those parts which are not considered edible.

Broccoli (*Brassica oleracea* var. italica) are commonly consumed in most parts of the world. Out of the entire broccoli plant (roots, stalk, leaves and flowers), only about 15% of it is eaten, while the rest is discarded. The stalk only, accounts for 21% of the total broccoli fresh weight, representing a huge waste, especially considering its theoretical edibility. While it's partially true that stalks are used in some households, industries that produce frozen ready-to-cook broccoli products, or cooked ready-to-eat products, surely throw the stalks away.

In this study we explored the possibility of re-utilizing broccoli stalks with the aim of obtaining a rich in antioxidants and probiotic product. In particular, we compared and assessed the effects of four thermal and physical disruptive treatments, being pasteurization, microwaving, freezing and autoclave (121 °C, 5 min), on the content of antioxidants measured as four different parameters (total phenolic content, total flavonoids content, DPPH assay and ABTS assay for scavenging activity). For microwave pre-treatment, 16 different combinations were tested (2, 4, 6, 9 W/g for 4, 5, 6, 7 min) to assess which one was the best performing in terms of phenols variation and microbial growth over 24 h. At the end, the 4 W/g 5 min combination was chosen.

The pre-treated broccoli were fermented using *Lactiplantibacillus plantarum* to see how antioxidant content and microbial population would mutate over the span of 96 h.

In a future development of the study, the possibility of reducing the fermented product into a powder by lyophilization will be assessed, with the aim of obtaining an antioxidant and probiotic functional product.

The study was entirely conducted in the laboratories of the department of Food engineering and development (Instituto de Ingeniería de Alimentos, FoodUPV) of the Universitat Politècnica de València. Laboratory activities were overviewed by Professor Cristina Barrera Puigdollers and Professor Lucía Seguí Gil. The project took part in a bigger work on the reutilization of agricultural and food processing waste coming from the handling of different Brassica vegetables.

1. Introduction

1.1. Food waste: data and importance.

Food waste is one of the biggest problems related to the whole world food chain, and its management is key to a more sustainable and efficient supply system. The loss of edible products can be linked to various stages of the supply chain, which can be summed up as the following ones: primary production, processing and manufacturing, retail and other distribution modalities of food, restaurants and food services and households. For what concerns Europe, the total food loss amount reached 59 million tons of fresh mass in 2020. It was shown that most of the food loss was related with how edible products were managed by consumers (households), making up for 53% (or 31 million tons) of the total amount of wastage. (EU, [1])

In 2019, a total of 931 million tons of food were wasted in the whole world, a number that can be compared with the latest measured value of produced goods, that belong to the year 2018, when 5.3 billion tons were available globally. In almost all countries, it's possible to observe how the most important sector when looking to food loss is the households compartment: 11% of the total available food was wasted at household level, followed by 5% in food service and 2% in retail. In the Food and Agriculture Organization report (State of Agriculture report 2019) (FAO, 2019[4]), it was stated that in the production step (including post-harvest and supply chain but excluding retailing), 14% of the total food production was wasted, with a peak of over 20% in Central and Southern Asia, and a minimum of around 6% in Australia and New Zealand (Fig.1). One of the biggest challenges is the recognition of a reliable and efficient way that would allow the most correct measurement of food waste possible. A new approach was explored in the UNEP Food Waste Index Report 2021: the Food Waste Index (FWI). It can be applied at three different levels depending on the available data. The first level of evaluation uses modelling for all those geographical areas in which data are not satisfactory, this will give

out an approximate value. The second level of evaluation is obtained by actively collecting data from each country, while the third level provides additional information that can suggest possible interventions with the aim of actively reducing the amount of food waste. Differently from the Food Loss Index (FLI), which is held by the Food and Agriculture Organization, which, as anticipated before, only takes in account losses along the supply chain but does not include retail and households, FWI also consider these last two steps of the chain, furthermore it includes direct reports from countries about the losses at manufacturing level, and for these reasons is considered more complete. The FWI is expressed as a number that goes from 0 to 100 and indicates the reduction of food wastage in respect of a baseline year (to be set in the future).

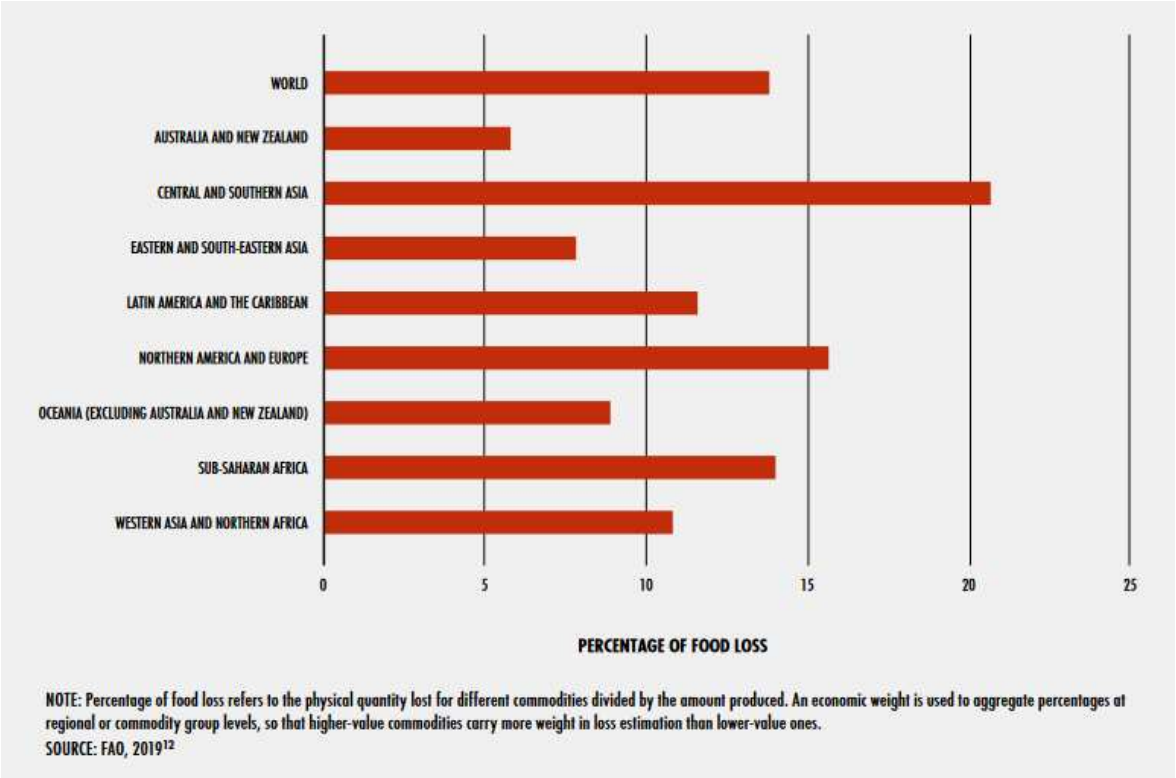


Figure 1. Food loss registered from harvesting to retailing in different geographical areas. FAO, 2019 [4]

Food loss has an impact on food security. The food demand is expected to grow by 50% over the next 30 years (Santeramo, 2021) so being able to make more food available, not only by increasing the production, but also by avoiding the waste of those goods which are already being processed along the food chain, becomes a fundamental action. Effects of food loss on food insecurity have already been assessed. For example, in a case study analyzed by Nyambo (2008), the improvement of storage facilities for maize and sorghum in Nigeria, severely decreased the loss of grains caused by insect infestation, partially increasing the food availability of local farmers who relied on the annual production for their own nutrition. But even in developed countries the problem of food waste impacts food security in low income communities. Eikenberry and Smith (2005) conducted a study on the phenomenon of dumpster dwelling in a North American urban area, and reported how frequent this habit is and how food waste that is directed to the dumpster could actually be used to fuel food security programs, in favor of the low-income part of society, which rely on this kind of support.

1.2. The institutional importance of Food security

The FAO, in their Policy Brief (June 2006, Issue 2) entitled "Food Security", defines the four fundamental components of the Food security concept, identified as:

- Food availability: availability of sufficient quantities of food of appropriate quality;
- Food access: access by individuals to adequate resources (entitlements) for acquiring appropriate foods for a nutritious diet;
- Utilization: utilization of food through adequate diet, clean water, sanitation and health care to reach a state of nutritional well-being where all physiological needs are met.
- Stability: access to adequate food at all times. They should not risk losing access to food as a consequence of economic or climatic crisis or seasonal events. (FAO [7])

Even though a huge effort by the scientific community can be testified by the several studies conducted on the topic, the concerns about food waste only recently started to

become relevant at institutional level as well. Several conventions have been carried out by the United Nations and several intentions have been proclaimed by the single member states, some events have set specific guidelines and objectives, to push and guide the decision-making process. Food security is a crucial concept regarding the accessibility of food to the whole population. The USDA categorize the problem distinguishing between:

- High food security: "no reported indications of food-access problems or limitations."
- Marginal food security: "one or two reported indications—typically of anxiety over food sufficiency or shortage of food in the house. Little or no indication of changes in diets or food intake."
- Low food security: "reports of reduced quality, variety, or desirability of diet. Little or no indication of reduced food intake."
- Very low food security: "reports of multiple indications of disrupted eating patterns and reduced food intake." (USDA ERS, [8])

The UN lists 19 milestones in the history of food security and nutrition issue management (UN Library, [9]), the first being the UN Conference on Food and Agriculture (1943). Some of them were conventions, with the aim of discussing the problem and proposing solutions to face the growing issue of food security, like the establishment of the World Food Programme, in 1961, or the UN Millenium Declaration. Others set full-on objectives and guidelines, usually requiring the constant progress monitoring, evaluated over the span of more than one year, like the Rome Declaration on World Food Security and World Food Summit Plan of Action, in 1996, which objectives were evaluated five years later, in 2002, and the more recent United Nations Decade of Action on Nutrition, with the aim of identifying and developing a work program based on the Rome Declaration and its Framework for Action.

Reducing food waste also plays an important role in reaching more than one of the 17 goals set by the 2030 Agenda for Sustainable Development (UN, [11]), proposed, again, by the UN in 2015 (Fig.2). The Agenda is a call for action by and to all countries, both developed and developing, to take serious steps to move towards the resolution of several problems, being social, economic and environmental. Each one of the 17 goals is then sustained by

publications and events, and on the UN website is even possible to check all the actions that have been implemented.

Food plays a key role in a lot of these points and can arguably influence almost all of them as it is central to human kind and society in terms of economy, agriculture and industry, health, nutrition, biodiversity, species preservation, climate, energy and even peace and inequalities. But it's evident how the reduction of food waste is of primary importance especially for the points relative to hunger, responsible consumption and production, and climate action.

Figure 2. The 17 goals set by the UN in the 2030 sustainability agenda. (FAO)

1.3. Edibility: a subjective problem

The UNEP Food Waste Index Report 2021 states important definitions that differentiate between food waste, inedible parts and edible parts of food waste. In particular, inedible parts are defined as: "Components associated with a food that, in a particular food supply chain, are not intended to be consumed by humans." And more importantly "What is

considered inedible varies among users […], changes over time, and is influenced by a range of variables including culture, socio-economic factors, availability, price, technological advances, international trade and geography.".

The concept of what's edible and what is not can have a huge impact on the amount of food wasted, since edible parts can, in fact, be part of the food loss, as defined in the report. The variability of the concept of what is edible and what is not is particularly evident in the households. As an example, the consumer decides whether a potato needs to be peeled or if the skin of poultry has to be removed before cooking or eating. On the other hand, in the food industry this concept is much more fixed, as the production of ready-to-cook and ready-to-eat goods are regulated and well defined by the company guidelines. In this case, frozen french-fries will most likely not maintain the peel, as well as the production of a chicken schnitzel requires the removal of the skin in the first place.

In the study conducted by Moreno et al. (2020) an interesting approach was used, comparing what is considered and categorized as edible by institutional standards, such as the United States Department of Agriculture (USDA) and the Waste and Resources Action Programme (WRAP) (USDA, [5]; WRAP, [6]), and what was actually discarded during food preparation in consumers houses. A total of 69 food items were selected, and the panel was asked to categorize the goods as edible or not. As a result, 59% (39 over 69) of the total amount of items consumed by the panel fell in the same categories of the USDA, WRAP and panel listings. It's important to consider that USDA categorization is more strict about the edibility of different parts of any item, for example, the white membrane covering the grapefruit pulp isn't considered adapt for human consumption. The major differences were found in the consumption of stalky vegetables, such as broccoli and cauliflower, which WRAP categorization considers fully edible, while USDA and panel don't. In general, panel and USDA percentages of edible foods within the group chosen resulted equal, at 55%, while WRAP guidelines showed a 63% of edible items and an arbitrary inclusive list, filled by the study conductors, showed a 71%.

1.4. Brassica oleracea var. Italica, an overview.

Broccoli (*Brassica oleracea,* var. *Italica*) stalks, averagely make up for the 21% of the fresh weight of the mature broccoli plant, and together with leaves (47%) and roots (17%) forms the considered "inedible" part of the vegetable, with a total amount of 85%, while the edible portion only accounts for 15% of the total mass (Li et al., 2022). There are some important differences between the different parts of the plant when it comes to nutrients.

As shown in Fig.3 the composition of primary metabolites varies a lot between the three different parts. In particular, it's possible to observe how florets are richer in aminoacids, while the stem presents a higher content of fatty acids, organic acids (in particular lactic acid, malic acid and maleic acid) and sugars (exception made for myo-inositol) (Liu et al., 2018).

Brassicaceae is one of the most significant families when it comes to glucosinolates content, which are extremely important for the organoleptic profile of the plants, but can also be broken down mainly by myrosinase enzymes into several degradation products.

For example, isothiocyanates that were found having several health-related properties like the induction of Phase II enzymes and induction of apoptosis and control of mitosis in cancerous cells (Johnson, 2002). The distribution of these substances in broccoli seems to be unbalanced towards the florets, as the total glucosinolates contents in florets, stems and leaf are respectively 34.66 μmol/g dry weight, 7.45 μmol/g dry weight and 10.08 μmol/g dry weight. 15 significantly expressed genes were individuated, showing a consistent presence in the whole plant, although apparently being promoted mainly in the floret region. Stem tissue was also found to be poorer in carotenoids and chlorophylls, in the overall content of mineral elements, in vitamin E and K content (most importantly when compared to leaves) (Liu et al., 2018).

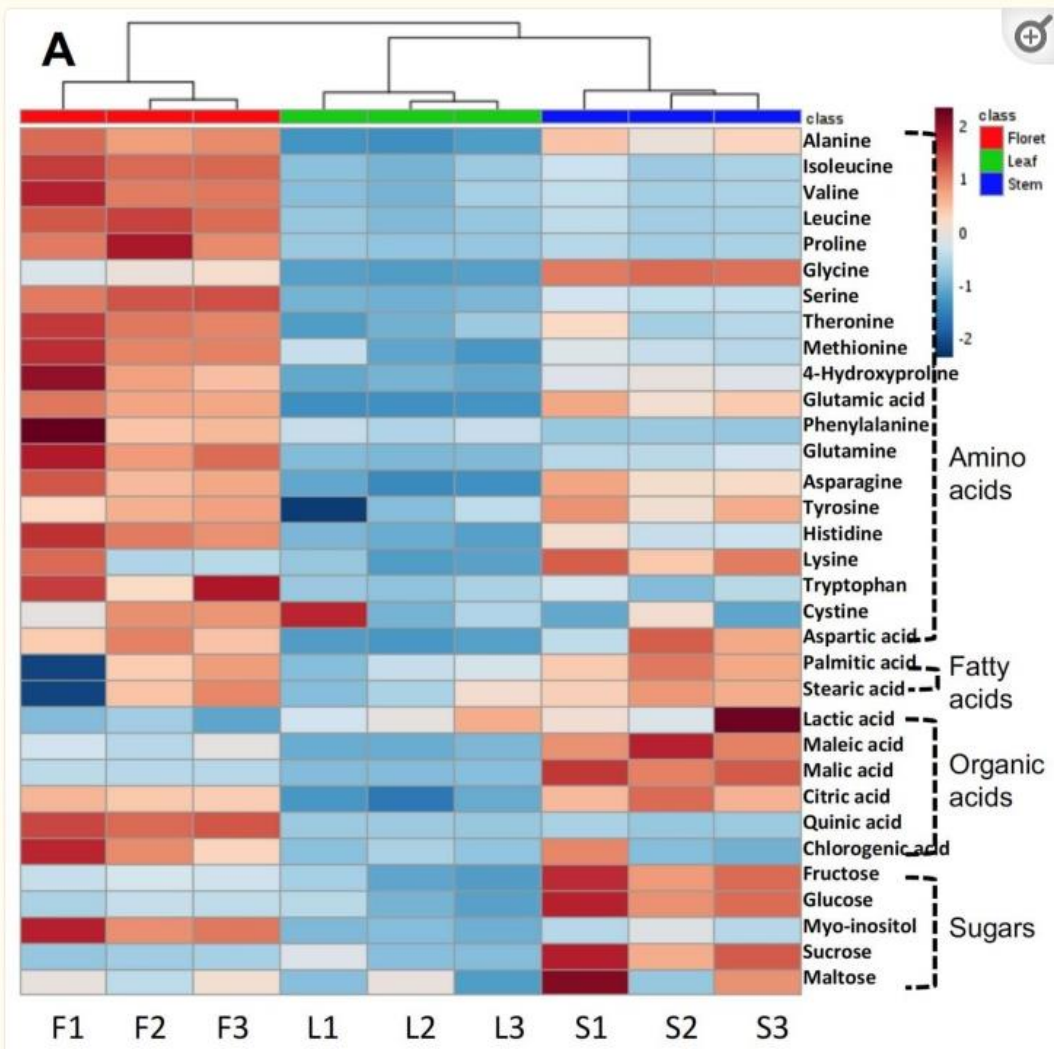


Figure 3. Expression map of primary metabolites across the three main parts of broccoli plant: floret (red), leaf (green) and stem (blue). (Liu et al., 2018)

Even though their nutritional content is not the best among the three parts, the nutritional quality of the stems can't be undervalued, and since they represent more than one fifth of the entire weight of the plant, they surely can't be ignored as a possible human consumption item. Antioxidant activity can also vary between the three different parts of the broccoli plant. Guo et al. (2001) explored the scavenging activity of stems, flowers and leaves, measured through the DPPH method, showed higher results in stems and flowers.

1.5. Antioxidants and antioxidant activity

Antioxidant activity has seen an increased interest from the scientific community over the last years. Nowadays, oxidative stress can be caused by several elements which are extremely common in our lifestyle, such as the ingestion of processed food, exposition to various chemicals and lack of exercise (Sharifi-Rad et al., 2020). In general, free radicals, which are the main actors of the oxidative stress, are produced in almost all metabolic processes, mainly as reactive oxygen species (ROS). They don't always play a negative role in the physiology of human body, as they participate, among other processes, in the destruction of non-self cells during the immune response and the apoptosis in human tissues. The human body is able to produce antioxidant enzymes, such as superoxide dismutase, glutathione peroxidase and catalase, and even to synthetize antioxidant molecules among which there are ascorbic acid, glutathione and vitamin A (Matés et al., 1999). However, the homeostatic condition in which the production of ROS is balanced by the self-antioxidative power, can be perturbated due to pathological conditions (auto-immune diseases, hypersensitivities such as food allergies and so on), or to a stress condition, in which the exposure to prooxidant molecules is higher than normal. If these conditions are maintained throughout a long period of time, they can ultimately lead to the disruption and/or modification of fundamental components of the cells, like lipids, proteins and nucleic acids, potentially causing an altered immune response, chronic inflammation and cancers (Sharifi-Rad et al., 2020).

Food is a great source of antioxidative molecules, as many are found in a large variety of fruit and vegetables (phenolic compounds, flavonoids, carotenoids, pectin, anthocyanins and all the derivative compounds). Their application in the food industry can spread from the enrichment of goods with additional antioxidants, the prolongation of shelf-life or, more simply, the intake of foods which are naturally containing antioxidants (Zehiroglu et al., 2019). The importance of intaking dietary antioxidants which are naturally contained in food, is enhanced by the fact that most synthetic antioxidants, although being highly stable and cheap, have shown remarkable toxicity and carcinogenicity, in addition of being

non-biodegradable. On the other hand, natural antioxidants don't show toxicity at the concentrations in which are normally contained in food and they don't need to be added during food processing as they are widely present across fruit, vegetables and edible plants (Narayana Saibaba, 2023).

Broccoli are not an exception as they contain several antioxidant substances. The content varies among the three main parts of the plant (stem, floret and leaves), but the use of the normally excluded tissues as a source of antioxidants can represent an effective solution to reduce waste during the processing step of this vegetable. In a study conducted by Jang et al. (2015), several antioxidant molecules were identified in broccoli. The largest component was isothiocyanates, of which sulfhydryl group can work as antioxidant while stimulating conjugating enzymes (Phase II enzymes of compounds metabolization) and phenols.

1.6. Antioxidant activity and pre-treatments

An important aspect of the antioxidant molecules is the stability and, more in general, the variation to different phenomena. Heat treatments are the most used treatments in food industry, with the aim of controlling the microbiological population and making the food edible. Natural polyphenols showed a higher heat stability in respect to synthetic ones (Volf et al., 2014), and in other studies, the total phenolic content even increased in response to heat treatment in different matrices (Jeong et al., 2004; Ross et al., 2011). Although it's possible that the increase can partially be due to the concentration of those compounds caused by the loss of water during the heating process. Anyway, a degradation of phenolic compounds and the reduction of antioxidant activity has been recorded at temperatures higher than 180°C (Ross et al., 2011). Similar studies were conducted on Broccoli, showing different results over the effect of heat treatments on antioxidant properties. López-Hernández et al. (2022) and Czarnowska-Kujawska et al. (2022) studied the effects of different cooking methods on broccoli antioxidant activity. In the first study, a decrease was clearly observed with boiling and microwaving, while in the second one, microwaving

decreased phenols and antioxidant activity during the first minute of treatment, while increasing it in the samples treated for 3 and 5 min. The increase in the concentration of antioxidant compounds can be due to loss of water from the sample.

Pre-treatments can also have other effects, that may be directly connected to the loss and acquisition of antioxidant power. All major cooking methods, like freezing, boiling, microwaving and blanching, produce some kinds of structural damages to vegetable tissues, like cells disruption and parenchyma fissuration, causing decrease in turgor, loss of water and loss of textures (Paciulli et al., 2016). Tissue damage can be linked to the release of some antioxidant molecules, as well as fermentable substances which are present in bound forms in raw vegetables (Gonzalez et al., 2010, Cömert et al., 2017)

1.7. Importance of fermentation and its application to *Brassica* plants.

Fermentation is one of the oldest ways to preserve foods and improve their organoleptic characteristics, and it dates back to about 13000 years ago (Liu et al., 2018). Beer was probably the earliest product obtained from fermentation and it represent a great example for all the improvement that this biological process can deliver, it's an effective way of preserving the nutritional properties of cereals throughout a long period of time, it can give positive organoleptic characteristic, the alcohol contained in the beverage can effectively control the microbial population, making it safer to drink, and finally it gives a recreational purpose which surely has had an important role in the spreading. During fermentation, a microbial agent will consume part of the substrate in an anaerobic environment, producing oxidized coenzymes that could otherwise not be produced and making up for the inoperability of the electron transport chain due to the absence of oxygen. An exception is acetic fermentation, which happens in an aerobic environment and involves the oxidation of ethanol to acetic acid. Fermentation is applied to an immense variety of substrates, ranging from animal derived products (meat, fish, milk) to potentially every plant. Also, the fermenting microorganisms vary among the substrate, their presence can be linked with

a natural occurrence on a specific substrate, i.e. lactose fermenting bacteria are more likely to already be present on a milky substrate, while on a fruit, which is rich in glucose and fructose, a range of alcoholic-fermenting yeast and acetic-fermenting bacteria can easily be found. Despite its ancient origin it has never lost importance in the food production sector, on the contrary, the study of fermentation has developed, this time with the aim of improving the final products, the efficiency and yield, selecting the best and/or new microorganisms and characterizing them using meta-genomic analysis or any other genomic tool. Due to this evolution, in order to achieve a better standardization of the process, fermentation has switched from a naturally occurring event to a human controlled one, by using previous batches to inoculate the new substrate or by introducing selected population of bacteria, yeasts or molds that have been well characterized from a genetic, genomic and metabolomic point of view. This way, the organoleptic and technological aspects of the production can efficiently be managed throughout the process. Yield of production and organoleptic changes are not the only traits that can be exploited. Other aspects have seen an increase in their importance, like the probiotic characteristic of fermented goods or the ability to produce bacteriocines. In the first case, probiotic bacteria gained a lot of importance in the scientific community, as they seem to participate in a vast range of process, playing an important role in the human gut. They have the ability to regulate receptor expression/repression, they can regulate other molecular processes such as the expression of proteins and the induction of microRNAs. Moreover, they were found to be participating in the regulation of the immune response both upregulating (i.e., induction of cytokine production) and downregulating (i.e., inhibition of signaling pathways related to various inflammatory processes) (Yousefi et al., 2019). Bacteriocines are small peptides produced by different bacteria, mainly lactose fermenting bacteria, that showed anti-bacterial properties. They are produced by both Gram-positive and Gram-negative bacteria and they are divided into different groups based on their molecular structure. Each group is also characterized by their activity against different microbial species. They have several pathways of action, some of them can interact with the cytoplasmic membrane of sensitive bacteria, creating ion channels that perturb the cell homeostasis, other can induce

the release of autolytic enzymes, leading to the cell lysis while bacteriocines of the lantibiotics group can inhibit the synthesis of the cell wall, more specifically of peptidoglycan. Gram-negative toxins can even directly cause DNA damage, being endonucleases, as is the case of colicin E2, produced by *Escherichia coli* and other Enterobacteriaceae (Karpiński et al., 2013).

For these reasons, nowadays fermentation is gaining more and more importance not only as a food-transforming process, but also as a functional and bioactive improvement of the food that has been fermented.

As such a versatile tool, fermentation has been exploited as a way to obtain added-value substances from agricultural and food waste. It has been applied to a wide variety of by-products, ranging from processing of starchy goods, such as potatoes, to ligneous residues, to obtain several new substances. Among others we find energy sources, such as bioethanol, biodiesel and hydrogen, or industrial molecules such as hydrolytic enzymes and aroma molecules. Three main techniques can be used: submerged fermentation (the process happens in a water-suspended environment), solid state fermentation (the inoculum is added directly to the substrate, coating the outer layer) and anaerobiotic digestion (bacteria operate in an anaerobiotic environment). Solid state fermentation seems particularly convenient as it is easier to operate and requires little to no water in the bioreactor, and despite being less controllable than the submerged method, may actually have a lower operational cost and a lower environmental impact (Hadj Saadoun et al., 2019). Solid state fermentation is also one of the oldest techniques used in fermentation and many examples can be found in traditional fermented foods all around the world. Brassica plants, cabbages in particular, are among the most fermented vegetables in the world, representing the main ingredient for preparations such as Sauerkraut (Europe and USA) (Fig.4a), Kimchi (Korea) (Fig.4b), Dhamuoi (Vietnam) and Gundruk (Eastern Himalaya) (Di Cagno et al., 2013).

Even though broccoli didn't see a use in fermentation as common as cabbages, their health-related characteristics, paired with fermentation, can offer a highly functional product, furthermore the application of fermentation may suggest new possibilities to recover parts

of the plants that would otherwise go wasted. Fermentation can have several effects on the properties of broccoli and broccoli stems, that vary from the improvement of the probiotic properties and bacteriocines content. Some strains (i.e. *LeviLactoplantibacillus brevis*, *LimosiLactoplantibacillus fermentum*, and *Lactiplantibacillus plantarum*) even showed the ability to enhance the bioactivity of some molecules such as glucosinolates and polyphenols, which have to be activated by the digestion carried out by digestive enzymes that may not hydrolyze completely this molecules (Iga-Buitrón et al., 2023). The health-related impact of a product rich in bioactive compounds such as broccoli, paired with fermentation and its advantages, might offer an interesting beneficial product, and the possibility of applying these principles to parts of plants which are not commonly eaten, can also have an effect on reducing food waste, mainly at industrial level, changing the concept of what is edible and what is not.

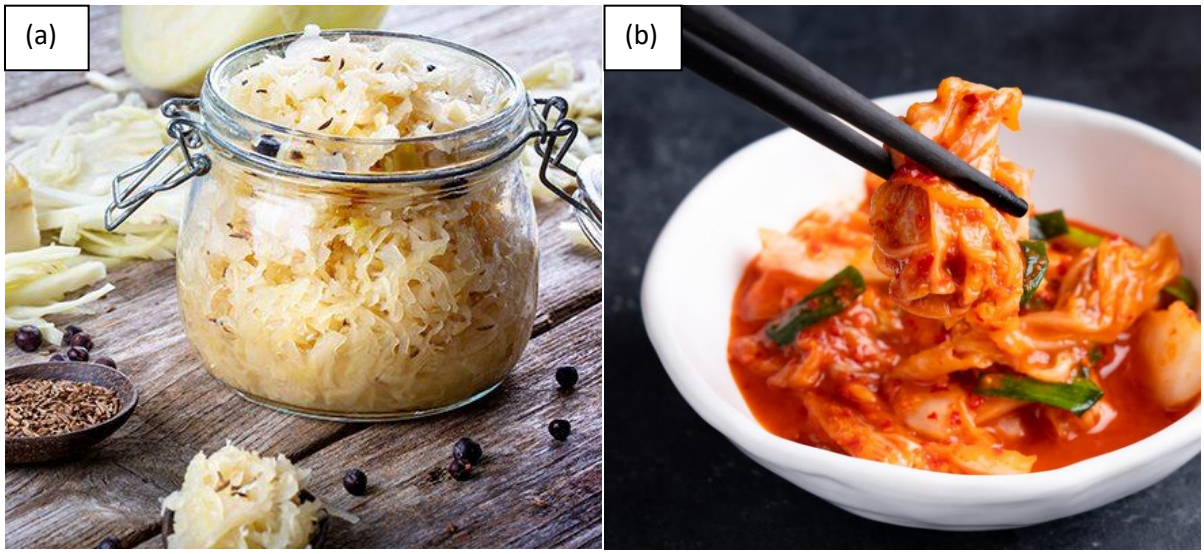


Figure 4a-4b. Two traditional fermented products obtained from Brassica plants, in these specific cases from *Brassica oleracea* cv. Capitata: *Sauerkraut* (Fig.4a) and *Kimchi* (Fig.4b)

1.8. *Lactiplantibacillus plantarum*

Lactiplantibacillus plantarum (formerly *Lactobacillus plantarum*) (Fig.5) is a widely spread lactic acid bacteria, as it is part of the microbiota of many food matrices such as dairy products, meat, fish and various fermented products, of animal feed, and animal and human gut microbiota. It is a Gram-positive bacterium, it can't produce bacterial spores, it is microaerophilic and mesophilic, although it can easily growth at temperatures between 10 and 15 °C. It is a facultative hetero-fermentative bacteria, hexose sugars are fermented and transformed into lactic acid, while pentose sugars can also be converted into acetic acid. It can also produce many other compounds, like diacetyl, acetoin and butanediol, which give typical aroma to some dairy products. Compared to other lactic bacteria, it has a lower proteolytic activity but higher levels of esterolytic and lipolytic enzymatic action, both intracellular and extracellular. It can produce bacteriocines, mainly plantarin C, plantaricin TF711 and pediocin AcH. It has also shown the production of some peptides and one protein with antifungal activity, as well as some antifungal hydroxylated fatty acids. It has been classified as probiotic microorganism since 1999 (Corsetti and Valmorri, 2011).

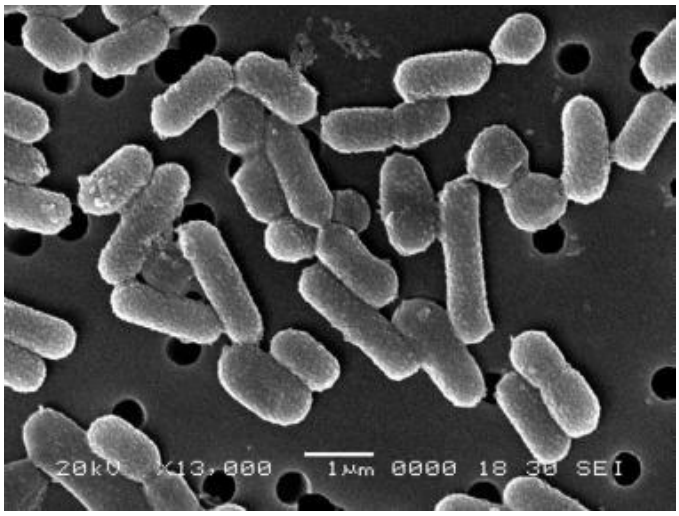


Figure 5. Micro-morphological image of *Lactiplantibacillus plantarum*. (Valan Arasu et al., 2015)

2. Aim of the study.

The study was entirely conducted in the Universitat Politècnica de València, the main university of the Spanish city of Valencia. In particular, the laboratory I've been part of was under the department "Instituto Universitario de Ingeniería de Alimentos (FoodUPV)" (Food Engineering and Development Institute), in the "Ciudad Politécnica de la Innovación" section of the campus (Fig.6). The study was revised by Professor Cristina Barrera Puigdollers and Professor Lucía Seguí Gil. The work took part in a bigger project on the reutilization of agricultural and food processing waste coming from the handling of different Brassica vegetables (grant number AGCOOP_A2021/020). In particular, for broccoli, the possibility of obtaining a functional powdered product from the pre-treatment and fermentation of the stalks was being studied. The powder form was obtained by partial dehydration and subsequent lyophilization, the microbial activity and antioxidant properties of the powdered product were then evaluated.

The aim of this project was to assess whether any pre-treatment could effectively produce a higher content of antioxidants, while at the same time increasing the concentration of fermentable sugars by breaking cellular structure and releasing them, thus increasing microbial growth. It also evaluated the role of fermentation by *Lactiplantibacillus plantarum* in the variation of the levels of antioxidants and antioxidant activity and microbial population over the span of 96 h, with these parameters being evaluated every 24 h.

The first part of the study was conducted exclusively on the microwave pre-treatment to assess the best combination of power and time, based on the final phenolic compounds content and microbial growth after 24 h of fermentation. The most efficient combination, supported by the data on phenolic compounds content and microbial growth, was selected.

The second part, which represented the main section of the study, was the comparative evaluation of the effects of four pre-treatments: pasteurization, autoclave pre-treatment (121 °C, 5 min), "domestic" freezing (-15 °C, overnight) and microwaving (4 W/g, 5 min).

The samples which underwent freezing and microwaving were also pasteurized, the protocols will be further described in the "Materials and methods" section. A 96 h fermentation was then initiated using *Lactiplantibacillus plantarum* as microbial agent. Every 24 h total phenolic content, total flavonoid content, antioxidant activity (DPPH and ABTS), pH and microbial growth were assessed, to evaluate which combination produced the best results.

Figure 6. Ciudad Politécnica de la Innovación, Universitat Politècnica de València (Source: UPV)

3. Materials and methods

3.1. Raw material.

The raw broccoli was bought from a local supermarket the day before the experience (in this case it was stored in the fridge at 4 °C), or the same morning. The flower portion of the broccoli was removed and the stalk was cut into pieces of about 1x1 cm dimension. The cubed sample was then treated with 6 to 10 mL/100 g of sample of a commercial 0.37 % (p/v) solution of sodium hypochlorite. The chunks would then be rinsed twice with tap water and drained. After the washing step the material was ground in a TM31 Thermomix® food processor (Vorwerk, Madrid, Spain) in batches of approximately 200 g, for 4 seconds at maximum speed. The ground stalk was scooped out of the mixer and distributed into 250 mL sterilized twist-off glass jars and was then pasteurized in a JP SelectaTM Precisdig thermal bath (ThermoFisher Scientific) until reaching an internal temperature of 82 °C maintained for 1 min.

3.2. Microbial strain.

The microorganism used for the solid state fermentation of broccoli stalks was *Lactiplantibacillus plantarum* strain CECT:749 from the Colección Española de Cultivo Tipo (CECT, Paterna, Spain). The inoculum was prepared from frozen microorganisms, which were regenerated in MRS broth for 24 h at 37 °C in a Selecta Incudigit incubator. After this first step of incubation, 0.1mL of the growth liquid, properly vortexed, were plated onto MRS agar, and re-incubated for additional 24 h, or until a visible layer of colonies was present on the surface of the medium. These pre-inoculum plates were sealed with parafilm and stored in the fridge (4 °C) for further use. To obtain the inoculum, a sufficient amount of microbiological material was picked from the plates and transferred into 9 mL of MRS broth, which was then left in the incubator for 24 h at 37 °C, until they

reached a microbial population of $(6 \pm 3) \cdot 10^9$ CFU/mL. After the incubation period the tubes were either used or kept in the fridge for no more than 72 h.

3.3. Conditioned material pre-treatment.

3.3.1. Microwave pre-treatment.

Microwave pre-treatment (MWPT) was analyzed for the whole first part of the study. As suggested by Conesa et al. (2016), we assessed the possibility of exploiting microwave treatment to increase the presence of reductive sugars such as glucose, therefore enhancing microbial growth during the fermentation step. We adapted the combination of power and time, accordingly to the microwave in our possession (Samsung GW72N), as follows: 100 W/50 g (2 W/g), 300 W/75 g (4 W/g), 300 W/50 g (6 W/g), 450 W/50 g (9 W/g) and 600 W/50 g (12 W/g). Each one of these powers were applied for 4, 5, 6 and 7 min. After the experimental trials we observed partial browning of the sample when 9 W/g were applied for 7 min and in all 12 W/g power-time combinations, so we finally selected the following microwave pre-treatments: 2, 4, 6 W/g for 4, 5, 6 and 7 min, and 9 W/g for 4, 5, and 6 min. The pasteurized jars were weighted before and after pasteurization so that, at the end of the pre-treatment, the water loss caused by the heating of the sample in the microwave could be reconstituted. To ensure a homogenous rehydration the content of the jar was thoroughly mixed.

In order to assess the best combination of power and time, different chemical analysis were carried out: total soluble solids content (TSS), total phenolic content (TPC) and reducing sugars content (RSC). In order to make the results more consistent, all the different concentrations were normalized for the dry matter (dm), which was calculated on the pasteurized broccoli. Water activity was also measured throughout all the samples.

The effects of MWPT on broccoli stalks tissue was analyzed through Cryo-SEM technique.

3.3.2. Other pre-treatments.

Two other pre-treatments were also applied to test their effects on the antioxidant properties, microbial growth and pH, being freezing and thawing (FPT) and autoclaving (APT). Freezing was operated on 250 mL glass jars containing 50 g of fresh broccoli. After 24 h in the freezer (-20 °C) the jars were taken out and placed in a thermal bath at room temperature, then gradually heated to prevent the glass from breaking, until pasteurization conditions were reached. APT was carried out in the autoclave (Systec VB-40) (Fig.7). Samples were pretreated at 121 °C for 5 min. The jars were then let cool at room temperature and used for the analysis.

Figure 7. *Systec VB-40* autoclave.

3.4. Inoculation and fermentation.

The fermentation was carried out in 250 mL glass jars with twist-off sealing. During MWPT testing, two jars for each combination of power and time were fermented separately for 24 h. For the comparative study of the 4 different pre-treatments the fermentation was studied along 96 h. Two jars were prepared and fermented separately for each pre-treatment to be analyzed every 24 h for the microbial growth, antioxidant properties and pH, for a total of eight jars for each pre-treatment. Inoculation was carried out by adding 1 mL of inoculum to 100 g of sample. Once inoculated, the broccoli stalks were mixed thoroughly, jars were closed and placed in the incubator at 37 °C to ferment.

3.5. Analytical determinations.

3.5.1. Water content and dry matter (dm).

Water content was measured by drying the broccoli stalk samples, which were weighted before the drying process, on glass capsules. The first stage of drying was operated in a stove (Selecta Conterm) at 60 °C for 24 h. After this pre-drying stage the capsules were moved to a vacuum stove (Selecta Vaciotem), which was operated at 0.2 bar and 60 °C of temperature. After approximately 48 h the capsules were extracted from the vacuum stove and weighted, the water content (x_w) and dry matter (dm) were calculated from the weight of the empty capsule (m_0), the weight of the fresh sample and capsule (m_1) and the weight of the capsule and the dried sample (m_2) as follows:

$$x_w = \frac{m_1 - m_2}{m_1 - m_0}$$

$$dm = 1 - x_w$$

3.5.2. Water activity (a_w).

The a_w was measured using an Aqualab 4-TE instrument at a constant temperature of 25 °C. Plastic capsules were filled with the sample until the whole bottom surface was covered and then placed in the instrument until the measurement was over. Two replicates were observed from each sample.

3.5.3. pH

The pH was measured for every fermented sample during the comparative study of the four pre-treatments, at 0, 24, 48, 72 and 96 h. In a beaker, 2.5 g of sample were suspended in 50 mL of distilled water and homogenized for approximately 10 min on the orbital shaker. The measurement was operated with a METTLER TOLEDO SevenDirectSD20 pH-meter. The instrument was firstly calibrated using a solution at pH 5.00. Once ready pH was measured directly by inserting the probe in the beakers.

3.5.4. Total soluble solids (TSS).

To measure TSS, 2 g of sample were transferred into a beaker and then suspended in 10 mL of distilled water. Beakers were then moved onto an orbital shaker, where the SS phase extraction was carried out for 30 min. At the end of the extraction step, the liquid phase of the aqueous solutions was analyzed for its °Brix in a refractometer (Atago 3T), at controlled temperature set at 20 °C (Polyscience temperature controller) (Fig.8). A sufficient amount of the solution was picked up with a Pasteur pipette and transferred on the refractometer glass. The measurement obtained from the instrument was then converted to mg TSS/g dm.

Figure 8. Atago 3T with Polyscience temperature controller for the determination of TSS

3.5.5. Dinitrosalicylic acid method for reducing sugar determination (DNS).

In order to assess the possible variation in the concentration of reducing sugars (RSC) due to the effect of the MWPT, the DNS method (Miller, 1959) was applied. The DNS reagent was prepared by mixing 30 g of sodium tartrate ($Na_2C_4H_4O_6$) and 1.6g of sodium hydroxide pellets (NaOH) in 100mL of distilled water. 1 g of dinitrosalicylic acid was finally added to the solution, while it was kept warm to facilitate the dissolution of the acid. The DNS reagent was kept in the fridge wrapped in aluminum foil for further use.

0.3 g ±0.001 g of sample were transferred into an Eppendorf microtube together with 1mL of an 80% (v/v) solution of ethanol in water, vortexed and left to extract for 30 min. Then the microtubes were centrifuged at 10000rpm for 10 min, the supernatant phase was collected and transferred into a test tube. The extraction phase was repeated again and the supernatant obtained from the second extraction step was mixed with the previous one. Two extracts were prepared from each sample. Another 2 mL of the 80 % (v/v) ethanol in water solution were added to the test tube. From each tube 500 µL of extract were transferred to another test tube together with 1mL of DNS, a blank was also prepared

mixing 500 mL of the 80 % (v/v) ethanol in water solution with 1 mL of DNS. The tubes were put into a thermic bath at 100 °C for 5 min and then immediately placed in an iced bath to suddenly reduce the temperature. In each test tube, 6 mL of water were added to dilute the analyte. The liquid was finally transferred into 3 mL spectrophotometry cuvettes and analyzed in a spectrophotometer (ThermoFisher Scientific Helios zeta UV-VIS) at 546 nm.

The curve was prepared using D-glucose (HPLC grade) in distilled water, so the results were expressed as mg of D-glucose per g of dry matter (mg GE/g dm), based on a standard curve prepared with HPLC-grade glucose (Sigma-Aldrich) in the range of 0-20 mg/mL, accordingly to the reducing sugar composition of fresh broccoli suggested by Bhandari (2015). The equation of the standard curve was the following:

$y = 0.0389x + 0.0524$ ($R^2 = 0.9944$), were y is the absorbance of the extract measured at 546 nm and x is its reducing sugars concentration in mg GE/g dm.

3.5.6. Antioxidant properties

Antioxidant properties were determined on extracts obtained by mixing 4 g of sample with 10 mL of a 80 % (v/v) solution of methanol in water (solvent). The mixture prepared in plastic beakers was agitated for 1 h on an orbital shaker in obscurity. After the agitation both liquid and solid phases were transferred into 50 mL falcon tubes and centrifuged in an Eppendorf Centrifuge 5804 R at 11000 rpm for 5 min at 4 °C. The liquid phase was collected after centrifugation and used for further analysis.

The total phenol content (TPC) was determined using the Folin-Ciocalteu method, which is the indirect measurement based on the redox reaction between the Folin-Ciocalteu reagent and the sample in an alkaline reaction environment. The reagent is mainly composed by a mixture of phosphomolybdate and phosphotungstate, which enhanced by a basic environment, will oxidize polyphenols to ortho-quinones, these semi-products will

then react by complexing the reagent itself and forming a chromophore substance, which colour tend to blue.

0.125 mL of extract, 0.125 mL of Folin-Ciocalteu reagent and 0.5 mL of distilled water were mixed in 3 mL spectrophotometry cuvette and let react for 6 min. Then 1.25 mL of sodium carbonate (Na_2CO_3) and 1 mL of distilled water were added. A blank was also prepared substituting the extract with 0.125 mL of the solvent. The mix was left reacting for 90 min in obscurity. At the end of the reaction absorbance was measured at 765 nm through a spectrophotometer (ThermoFisher Scientific Helios zeta UV-VIS) (Bas-Bellver et al. 2022).

The absorbance data were converted into mg of gallic acid per g of dry matter (mg GAE/g dm) with a standard curve prepared with gallic acid in the range of 0-600 mg/L. The equation of the standard curve was $y = 3.2877x + 0.1564$ ($R^2 = 0.9889$), where y is the absorbance of the mixture measured at 765 nm and x is the gallic acid concentration in mg/mL.

Total flavonoid content (TFC) was analyzed via the aluminum chloride assay (Christ et al., 1960; Ghatak et al., 2015; Shraim et al. 2021), which involves the reaction between $AlCl_3$ (aluminum chloride) and flavonoid molecules. In particular, the formation of Al(III)-flavonoids chelates will generate a yellow colour and the absorbance can be read at 368 nm. The $AlCl_3$ solution was prepared at 2 % in 100 mL of pure methanol (HPLC-grade) under a chemistry hood due to the highly exothermic reaction and mixed until completely dissolved. The 3 mL spectrophotometry cuvettes were then prepared with 1.5 mL of extract and 1.5 mL of the $AlCl_3$ solution and left reacting for 10 min. A blank was also prepared by substituting the extract with 1.5 mL of the solvent. After this time the cuvettes were analyzed at 368 nm in the spectrophotometer. The absorbance data were converted into mg of quercetin equivalents per g of dry matter (mg QE/g dm) using a standard curve prepared with quercetin in the range of 0-200 mg/L. The equation of the standard curve was the following: $y = 11.755x + 0.0915$ (R^2=0.9954), where y is the absorbance of the mixture measured at 368 nm and x is the quercetin concentration in mg/mL.

Antioxidant activity of broccoli extracts was evaluated through their scavenging activity towards ABTS and DPPH free radicals.

ABTS assay is a free radical based assay used to test the scavenging activity of an analyte. (Miller et al., 1997; Ilyasov et al., 2020) It's a Trolox equivalent antioxidant capacity test (TEAC) which assess the ability of the analyte to inhibit the radical activity of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical or ABTS•+, which is also a chromogen molecule absorbing at different wavelengths: 415, 645, 734, and 815 nm. During this study 734 nm was the wavelength adopted. The ABTS•+ was originally synthetized by the reaction between ABTS (2,2′-azino-bis(3-ethylbenzo-thiazoline-6-sulfonic acid)) and myoglobin, while nowadays is commonly obtained through the reaction between ABTS and potassium persulfate. In this study the ABTS•+ radical was obtained by preparing a 7 mM aqueous solution containing 0.0331 g of potassium persulfate, 0.192 g ABTS and 50 mL of distilled water, in obscurity. The solution was then kept in the fridge wrapped in aluminum foil for at least 16 h, or until eventual use. A potassium buffer was prepared by mixing 1.29 g of sodium phosphate monobasic anhydrous (NaH_2PO_4) and 5.72 g of di-sodium hydrogen phosphate anhydrous (Na_2HPO_4) in 50 mL of water until completely dissolved. The ABTS•+ reagent was progressively mixed with the potassium buffer until the absorbance at 734 nm reached 0.7 (blank).

0.1 mL of extract and 2.9mL of blank were mixed in spectrophotometry cuvettes, after 7 min of reaction they were analyzed at 734nm.

The absorbance values were converted into mg of Trolox equivalents per g of dry matter (mg TE/g dm) using a standard curve, which was constructed using serial dilutions prepared with 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (TROLOX), in the range of 0-0.2 mg/L. With the data obtained, a standard curve was built having the following equation and R^2: $y = -0.0795x + 0.7877$ ($R^2 = 0.9963$), where y is the absorbance of the mixture measured at 734 nm and x is the trolox concentration in mg/mL.

DPPH assay is another quantification method based on the scavenging activity of the analyte, this time towards the radical DPPH• (α, α-diphenyl-β-picrylhydrazyl) (Blois, 1958,

Kedare et al. 2011). DPPH• doesn't need to be activated, but due to its low solubility in water is commonly dissolved in alcohol. In this study a 0.1 mM solution was prepared mixing 0.0039 g of DPPH• into 100 mL of HPLC level methanol in obscurity conditions until the complete dissolution of the powder. DPPH• is not stable enough to be kept throughout the week so the unused solution was disposed after the single use.

0.1 mL of extract and 2.9 mL of the methanol:DPPH• solution were mixed in 3 mL spectrophotometry cuvettes. The blank was also prepared substituting the extract with 0.1 mL of the 80 % (v/v) methanol in water solution. After 60 min of reaction the cuvettes were analyzed at 517 nm. The absorbance was converted into mg of Trolox equivalents per g of dry matter (mg TE/g dm). The standard curve was prepared using serial dilutions prepared with 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (TROLOX), in the range of 0.125-0.2 mg/L. The standard curve had the following equation:

$y = 1.8061x + 0.0064$ ($R^2 = 0.9913$), where y is the percent inhibition of absorbance and x is the Trolox concentration in mg/mL.

3.5.7. Cryo-SEM electron microscopy.

Cryo Scanning Electron Microscopy, or Cryo-SEM, is a microscopy technique that require the freeze-drying of the sample. It has often been applied to plants, food and vegetables observation (Préstamo et al., 2004; Pathan et al., 2010). Cryo-SEM can be a very useful tool to assess the morphology of the surface of a vast variety of samples, including the composition of the tissue and its structure: a SEM instrument detects electrons that are reflected or knocked off through three different detectors, this makes up for a highly reliable image of the surface of the analyte, rather than the inside which is better modeled by the Transmitted Electron Microscope (TEM). The correct protocol requires the use of a conductive material, which can improve the contrast of the final image. The vacuum condition is also very important to obtain a clear enough image, because it will cause the sample to dehydrate more quickly, thus reducing the damage to the tissue. The analysis

workflow contains some major steps: preparation of the sample and placement on a stub, rapid freezing of the sample, creation of the vacuum condition, optionally the sample can be fractured to observe how the tissue responds to a cutting action, sublimation and conductive coating.

For the Cryo-SEM analysis we were hosted by the Departamento de Física Aplicada of the Universitat Politècnica de València. The instrument was composed of a body *(*Zeiss Ultra 55) and a sublimation chamber (Quorum PP3010) (Fig.9).

The differences observed in the tissual structure were assessed in the following samples: fresh broccoli, pasteurized broccoli, 2 W/g 2 min (mildest treatment), 9 W/g 6 min (toughest treatment) (all samples treated with MW were previously pasteurized). The sample was fixed on a sample holder using a cement prepared mixing colloidal graphite and Tissue-Tek® O.C.T. Compound, which was spread along the contact surface between the sample and the holder. Some strings of cement were also drawn from the top of the sample portion to the holder, in order to improve the conductivity making up for better contrast on the image. While the sample was being prepared, liquid nitrogen was poured into a vacuum barrel where it reached -196 °C, solidifying. At this point, the sample was picked using an insertion rod, which has a screw at the end allowing the transferring of the holder. The sample was inserted into the vacuum chamber, passing through a transferring chamber. When the vacuum was restabilized and the sample was completely frozen, it was moved into the sublimation chamber, while being kept inside the transferring chamber which guarantee the maintenance of a low pressure. Before being inserted, vacuum conditions were created into the sublimation chamber (pre-vacuum), then the sample was ejected from the transferring chamber and finally fixed onto a surface into the sublimation chamber. At this point the sample was dehydrated through sublimation, which was applied at -90 °C and 10^{-7} mbar throughout 15 min of time. At the end of the sublimation the sample was covered with platinum, this step is called quorum sputtering. The quorum sputtering lasted 30 seconds (if conductive strings were placed from the top of the sample to the holder, otherwise an additional 45 seconds needed to be added), at the end of which the

sample was moved to the observation chamber. From here the samples were observed through a monitor.

Figure 9. Cryo-SEM microscopy composed by the Zeiss Ultra 55 body and the Quorum PP3010 sublimation chamer.

3.5.8. Microbial growth (MG).

Lactiplantibacillus plantarum content in both the inoculum and in the fermented broccoli was estimated through microbial count on MRS agar plates. The inoculum was collected from the growth tube, 1 mL was transferred into another tube containing 9 mL of peptone water. For the inoculum, serial dilutions were prepared up until the 10^{-8}. 0.1 mL of the three most diluted suspensions were plated onto MRS agar plates and let incubating for 24-48 h at 37 °C.

For the evaluation of microbial growth in fermented samples, 1 g of broccoli was transferred into a stomacher bag together with 9 mL of peptone water, the bag was then placed into a stomacher (IUL instruments masticator) for 40 seconds to homogenize the suspension. From the bag, 1 mL of the suspension was transferred to a test tube containing 9 mL of peptone water, serial dilutions were prepared up until the 10^{-7}. The three most

diluted suspensions were plated onto MRS agar plates and let incubating for 24-48 h at 37 °C.

3.5.9. Statistical analysis.

Statistical analysis was carried out with the Statgraphics Centurion XVI program (Statgraphics Technologies, Inc., The Plains, VA, USA) by means of simple and multivariate analysis of variance (ANOVA) with a 95% confidence level.

4. Results and discussion

4.1. Effect of thermophysical pre-treatments on the properties of broccoli residues.

4.1.1. Preliminary study on microwave pre-treatment

Fresh and pasteurized samples were characterized for the following parameters: water content (x_w), water activity (a_w), total soluble solids (TSS), reducing sugar content through. DNS method (RSC) and the total phenols content (TPC). The data obtained are shown in Table 1. A considerable decrease in phenolic compounds content is observable, most likely due to the degradation of the molecules connected with the thermal treatment, which destroyed all phenols characterized by heat instability. It's also possible to observe an increment in the TSS content after pasteurization, that may be connected with the partial loss of water and the release of bound substances due to the action of high temperatures. Probably for the same reasons, also RSC increased after pasteurization.
Both water content and water activity didn't show any significant variation before after pasteurization.

Dry matter, which resulted as 0.076 g dm/g of broccoli, was then used throughout the whole study to normalize the results.

Table 1. Water content, water activity, TSS, RSC and TPC are shown for their variation between the fresh samples and the pasteurized ones. Error represented as mean standard deviation of the replicates.

Parameters	Fresh	Pasteurized
x_w (g H_2O/g)	$0.9282 \pm 0.0009_a$	$0.924 \pm 0.005_a$
a_w	$0.998 \pm 0.004_a$	$0.994 \pm 0.003_a$
TSS (mg TSS/g dm)	$0.70 \pm 0.022_a$	$1.30 \pm 0.03_b$
RSC (mg GE/g dm)	$20 \pm 1_a$	$27.91 \pm 0.07_b$
TPC (mg GAE/g dm)	$8.5 \pm 0.6_b$	$2.9 \pm 0.5_a$
Different letter in the same row indicates statistically significant differences with a 95% confidence level (p-value<0.05)		

RSC resulted significantly higher in the pasteurized sample compared to the fresh sample, this could be due to the action of heat that can disrupt some of the cellular structures, thus releasing reducing sugars.

Water activity didn't show significant changes among the samples, including the pasteurized ones (without MWPT), settling at 0.994 ± 0.002, also considering that, after every MWPT, the matrix was re-hydrated until reaching the pre-microwaving weight.

Pasteurized samples treated with microwaves (MWPT), with all the combinations possible obtainable from the powers 2, 4, 6 and 9 W/g and the times 4, 5, 6, 7 min (exception made for 9 W/g and 7 min), were characterized for the following parameters: a_w, TSS, RSC and TPC.

In Figure 10, the variation of TSS among samples pre-treated with different MW combinations is displayed. It's possible to observe several significant differences throughout the different samples. In all cases a low variation is shown after the first 4 min of treatment. In this range of time, milder treatments (2 and 4 W/g) seemed to induce a

slight positive variation while harsher treatments (6 and 9 W/g) showed a sensible decrease. Both 4 W/g and 6 W/g treatments showed a statistically significant decrease in TSS with the treatment time increasing from 4 to 5 min. Since the main components of the soluble solids fraction are carbohydrates, acids and nitrogen compounds (Ivanova et Al., 2021), it's possible that this amount of time is enough to degrade some of those compounds, for example due to non-enzymatic browning and proteins denaturation, but not to disrupt cells structures and liberate bound substances. This might happen in the 6 and 7 min treatments, as an increase in TSS is recorded. On the contrary, TSS content in the 2 W/g and 9W/g didn't seem to be affected by the increase of treatment time. This may be due to a balance between the degradation and the release of SS components from cellular structures. 4 W/g, 4 min MWPT combination seems to be the most efficient in terms of soluble solids, as it shows a relatively high positive variation with the lowest treatment time.

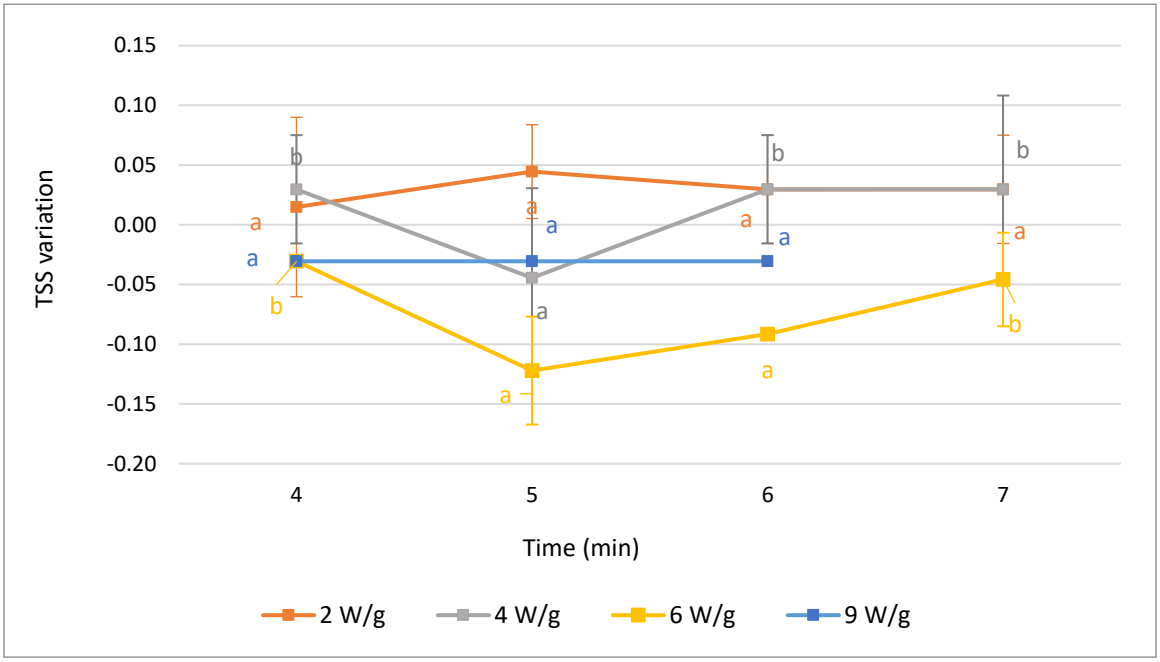


Figure 10. Effect of power and duration of MWPT on the variation in content of TSS in broccoli stems. Error bars represent standard deviation between replicates. Different letters with same colour indicate statistically significant differences with a 95% confidence level (p-value <0.05) within the same series.

In Figure 11, RSC variations are displayed. All samples and all treatments showed a decrease in the content of reducing sugars, such as glucose, fructose and galactose. Furthermore, all samples showed a consequent decrease with the increasing pre-treatment time. Little to no significant variations were recorded among different powers at same treatment times. This phenomenon may be due, again, to the degradation of sugars caused by non-enzymatic browning and other degradative processes enhanced by high temperatures presumably reached during microwave treatment (Cardoso et al., 2023; Eggleston et al., 2008). Of all the combinations at trial, 2 W/g for 4 min showed a less important variation in respect to the pasteurized sample seemingly representing the most efficient treatment. However, due to the net negative variation over the non-treated value, it's possible to state that MWPT doesn't have any positive effects on the total content of reducing sugars.

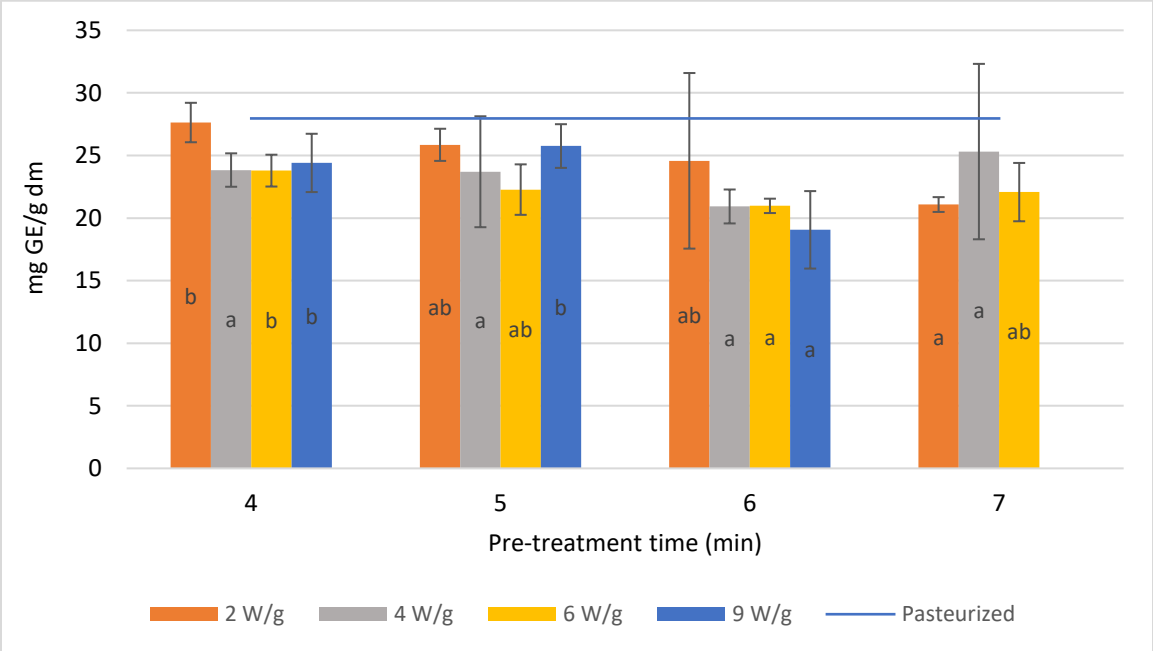


Figure 11. Effect of power and duration of MWPT on the variation of RSC in broccoli stems. Error bars represent standard deviation between replicates. Different letters indicate statistically significant differences with a 95% confidence level (p-value<0.05) within the same series.

Figure 12 shows the variation in content of the total phenolic substances (TPC) before and after MWPT. All combinations induce a decrease in phenolic substances, most likely due to the heat effect as irradiation can increase the temperature above the boiling point, thus enhancing the degradation (Liazid et al., 2007). A significant increase of TPC in respect to the 4 min trials is recorded at 5 min in both 4 W/g and 9 W/g, resulting in a minor decline of TPC compared to the pasteurized samples. This may be reconnected with the tissue disruption operated by massive water evaporation. The highest values are reached by the application of 4 W/g for 5 min, which almost reached the original content obtained for pasteurized samples. The 2 W/g and 6 W/g didn't show any particular variation, showing relatively low TPC. Also in this case, MWPT doesn't seem to positively affect the TPC, thus not being a convenient treatment for increasing this parameter.

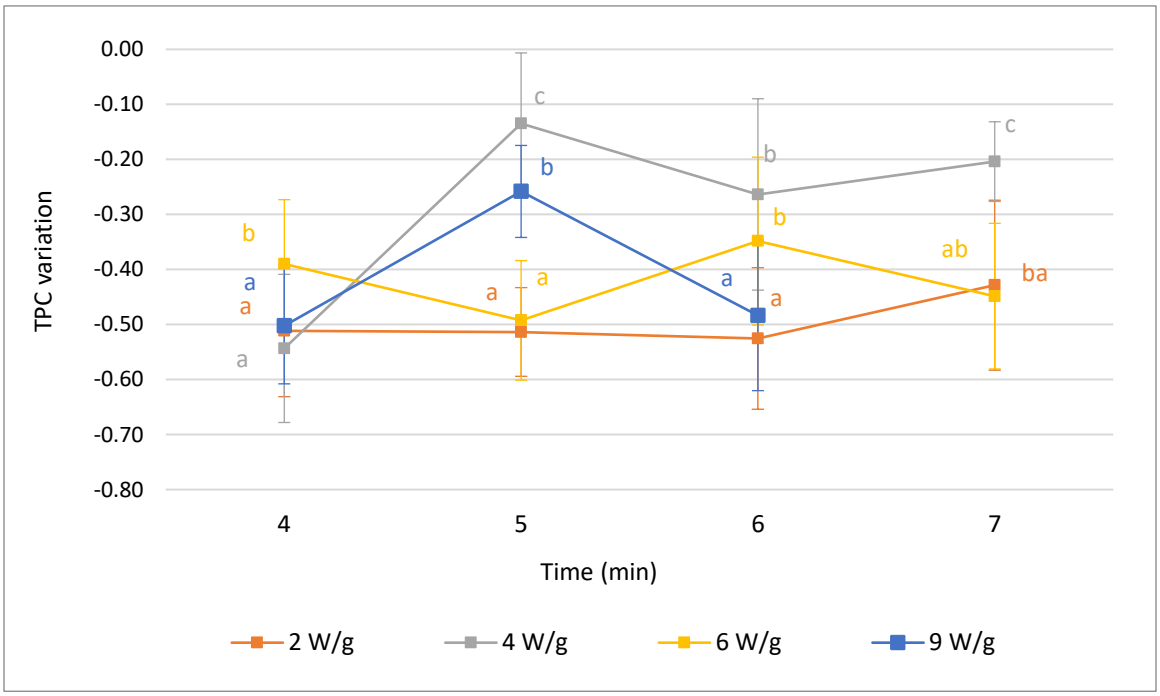


Figure 12. Effect of power and duration of MWPT on the variation of TPC in broccoli stems. Error bars represent standard deviation between replicates. Different letters with same colour indicate statistically significant differences with a 95% confidence level (p-value<0.05) within the same series.

Figure 13 represents the microbial growth (MG) in the samples as absolute variation in respect to the t_0 inoculated ones. Values are expressed in a logarithmic scale. Positive MG was recorded in all samples after 24h of fermentation. The highest MG was registered in the 9 W/g treated samples, which showed a greater rate than other treatments in all combinations. This may be due to a particularly high microbial population we observed in the starter used for the inoculation of the 9 W/g series samples, that may have led to slightly higher results in regard to the microbial growth. Both 4 W/g and 6 W/g treated samples showed a significant increase in microbial population as MWPT duration increases to 5 min, with the highest value reached by 4 W/g samples. 2 W/g pre-treated samples showed a progressive decrease in population as treatment increased in time. The comparison of microbial growth between MWPT samples and pasteurized samples will be deeper analyzed in the second part of the study.

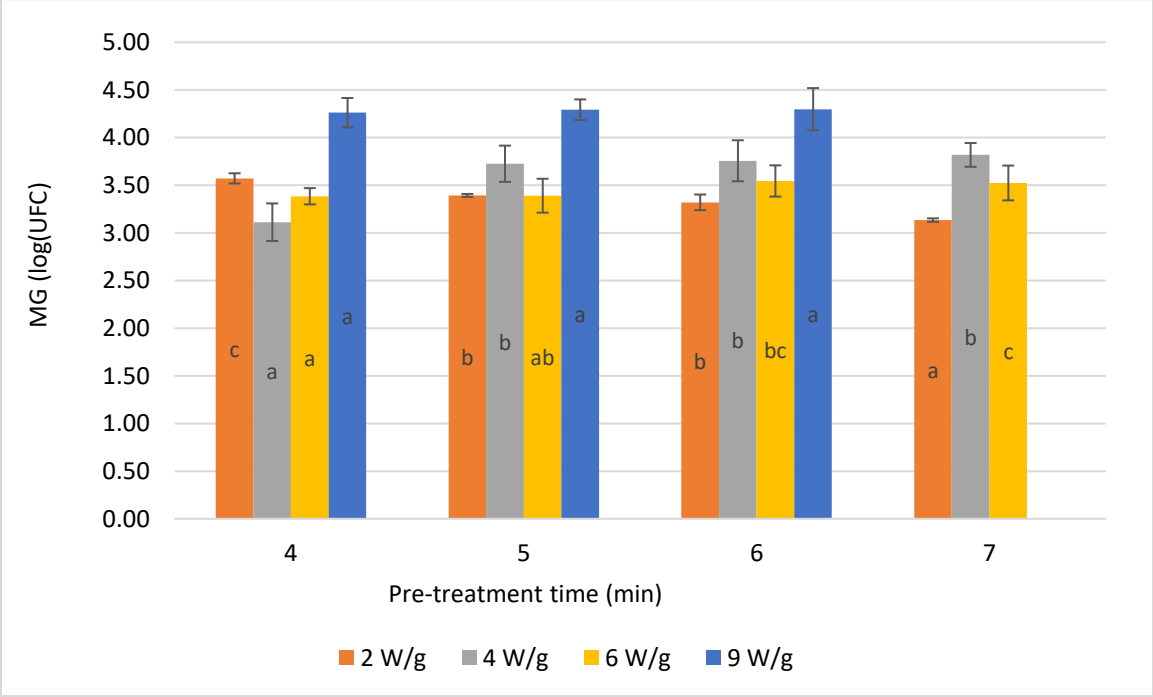


Figure 13. Effect of power and duration of MWPT on the growth of *Lactiplantibacillus plantarum* in broccoli stems. Error bars represent standard deviation between replicates. Different letters indicate statistically significant differences with a 95% confidence level (p-value<0.05) within the same series.

The endpoint of this first section of the study was to assess the most efficient pre-treatment that would lead to a higher MG with a minimum loss of TPC. Phenolic content generally decreased after MWPT, the 4 W/g for 5 min combination had the lowest negative impact. MG was observed to be higher in the 9 W/g pre-treated samples, although, considering the high power implied in this kind of treatment and the possible variability due to an originally higher microbial activity already registered in the inoculum, the 4 W/g pre-treatment might be the most efficient one, showing the highest MG among all the other combinations. Also, for a matter of time efficiency, and considering the non-significative variation of MG values in 4 W/g from 5 min to 7 min, it's possible to affirm that the best combination is 4 W/g 5 min.

Despite the negative effect of MWPT on TPC and the overall low variation in comparison with pasteurized samples, it was decided to keep this pre-treatment to be further characterized, to assess its effect on flavonoids, antioxidant activity and MG over longer fermentation times and to serve for the comparative study on the other pre-treatments.

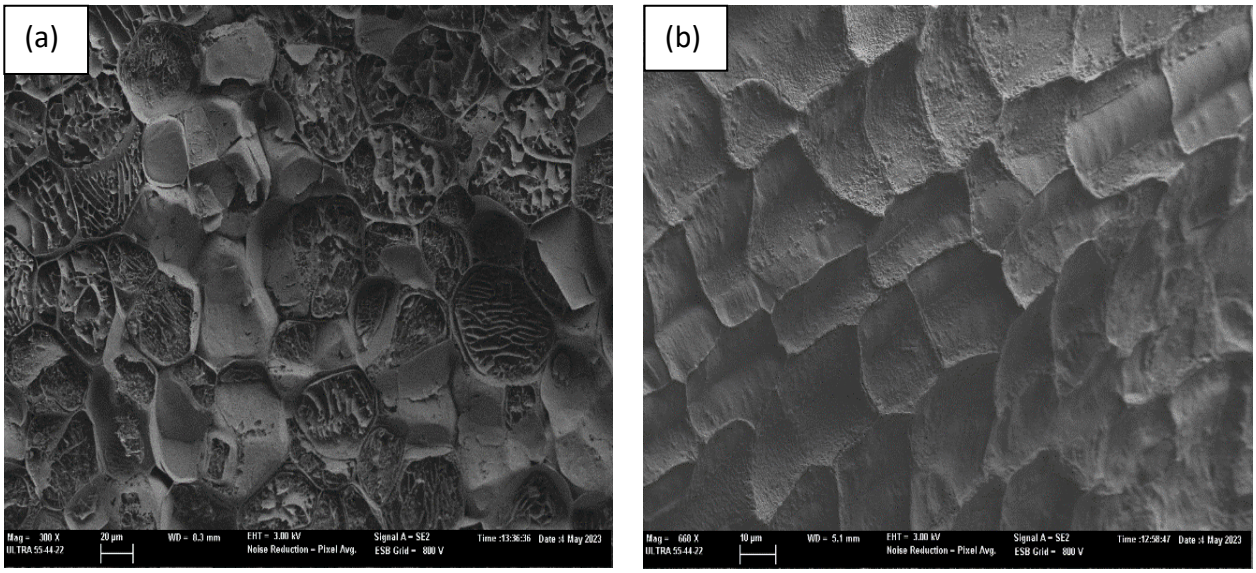
4.1.2. Cryo-SEM observations.

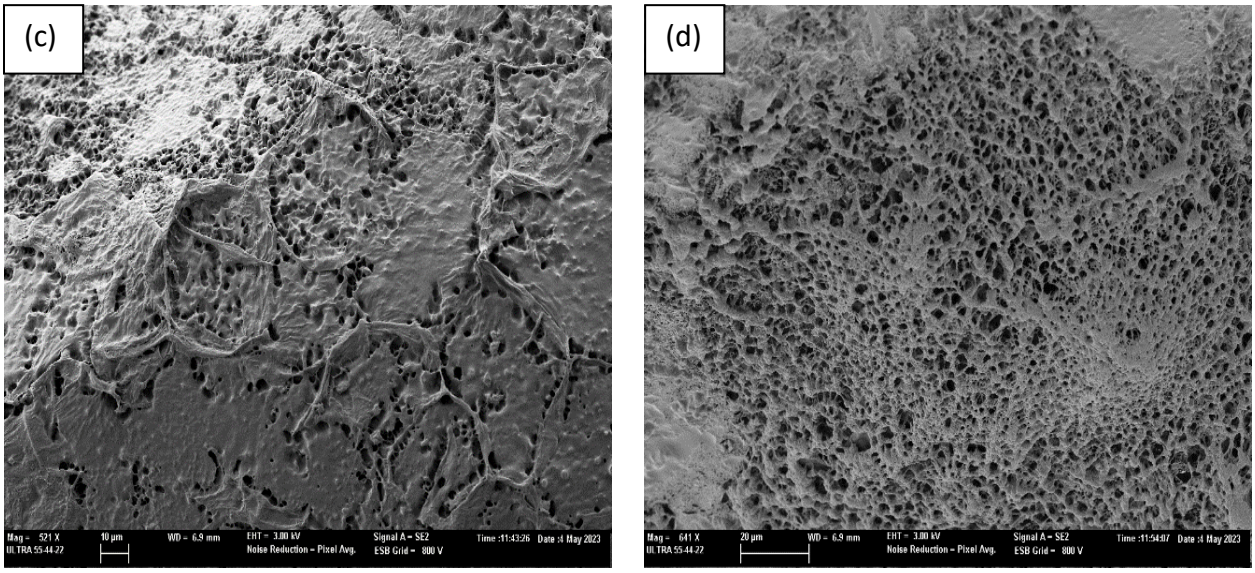
MWPT samples were also supposed to be analyzed through Cryo-Scattered Electron Microscopy. Due to technical problems with the instrument, only two broccoli samples were finally observed: a fresh stalk tissue sample (Fig.14a and Fig.14b) and a MWPT sample (2 W/g for 4 min) (Fig.14c and Fig.14c). The aim of this observation was to assess the disruptive effects of MWPT on the vegetable tissue, with particular focus on the integrity of cell structures, mainly cell walls.

An evident difference can be spotted in Fig.14 between the two samples. In the first couple of images, it's possible to observe a still pretty well distinguishable system of cell structures. Cell walls are well defined and mostly intact. The cells that were partially separated during the grinding process offer a view of the inside, in which several channel-like structures can be spotted. This is mostly due to the rapid vacuum freeze-drying that is

required for the samples to be observed (Wightman, 2022). Pasteurization may also have contributed to the formation of such artifacts, due to partial evaporation of the intra-cellular water contained in water-rich compartments, such as vacuoles (Gonzalez et al., 2010). However, the pre-treatment wasn't powerful enough to affect the structural integrity of the cell wall.

In the second two images, the surface of the sample shows a dense distribution of "holes" in the tissue, to the point that in both examples no intact cellular structure can be distinguished, if not partially in the first picture. In this case, artifacts produced by the violent evaporation of intracellular water created channels through the cell wall, disrupting its structural integrity and potentially releasing cells content in the extracellular space (Gonzalez et al., 2010). This process should increase the availability of nutrients, both carbohydrates and nitrogenous substances, among others. The disruption of tissues could also free some antioxidant molecules from a bound form (Cömert et al., 2017), although, as we've seen for the phenols content, this effect may be outbalanced by the degradation of such substances.





Figures 14: Fig.14a and Fig.14b were taken on the pasteurized sample, the magnifications are respectively 300X and 660X. Fig.14c and Fig.14d were taken on the MWPT sample, the magnifications are respectively 521X and 641X.

4.2. Comparative study on the different thermophysical pre-treatments.

Autoclave pre-treatment (APT) and freezing and unfreezing pre-treatment (FPT) were tested for their effect on the content of reducing sugars (RSC). The results were respectively $29_a \pm 2$ mg GE/g dm and $28_a \pm 1$ mg GE/g dm. No significant differences were spotted with the two treatments regarding the content of soluble sugars, and no particular decrease or increase has been recorded in respect of the pasteurized sample. However, both values are higher than those obtained from the fresh sample (20 ± 1 mgGE/g dm) and from all the MWPT samples with any power-time combination reported in the first part of the study. The selected microwave pre-treatment (4W/g for 5 min) and the pasteurization have been tested again for the RSC together with APT and FPT. This time MWPT samples

showed a significantly higher value, being 29 ± 1 mgGE/g dm, while pasteurized samples showed a more similar RSC, but still statistically higher than the value reported in the first part of the study (28 ± 2 mgGE/g dm). Broccoli demonstrated quite a high variability in almost all parameters, especially when trials were executed at different times of the year, due to the change of varieties and the differences in meteorological conditions. For this reason, results obtained from experiments operated at distant times can be hard to compare. If we consider the latest results, which is more convenient as the raw material is homogeneous among all treatments, it's possible to affirm that no significant variation is spotted between samples which have undergone different pre-treatments.

As explained in the materials and methods section, samples submitted to different thermophysical pre-treatments were analyzed for microbial growth, TPC, total flavonoids content (TFC), antioxidant activity through DPPH and ABTS assay (DPPH, ABTS) and pH, at 0, 24, 48, 72 and 96 h of fermentation.

Figure 15 shows the microbial growth along 96 h of fermentation. In all cases microbial growth was recorded after 24 h of fermentation, showing that broccoli can be considered as a good substrate for the growth of *Lactiplantibacillus plantarum*. The microbial counts remained constant, with little to no significant changes between 24 and 48 h. Only FPT and MWPT showed a small significant increase in microbial population. Over the following 48 h all samples showed a decrease in microbial population, most probably due to a lack of nutrients and overpopulation of the substrate. FPT samples showed a steeper decrease in microbial population, this is probably due to a casual variation, considering that no disruptive treatment seemed to affect the concentration of fermentable sugars.

Figure 16 displays the variations in content of phenolic substances (Fig.16a), antioxidant activity (ABTS and DPPH) (Fig.16b, Fig.16c respectively) and flavonoids content (Fig.16d). All samples showed a net decrease of TPC and DPPH antioxidant activity with respect to the fresh samples. The ABTS assay showed a very small increase of values in the autoclaved sample in respect to the fresh sample in the non-fermented sample, while all the other samples remained at a lower level. In general, samples treated in the autoclave

showed higher contents of antioxidant species and a higher antioxidant activity compared to all the other pre-treatments. The harsher conditions at which this samples were exposed might have increased the tissue damage, thus liberating more antioxidant molecules. High pressure cooking already showed to have variable action on antioxidants concentration different vegetables, displaying a less degrading effect compared to microwaves cooking in broccoli (Jiménez-Monreal et al., 2009).

TFC (Fig.16d) is the more interesting among the four parameters, as all pre-treated samples e$_x$hibited higher TFC compared to the fresh matrix at all stages of fermentation, exception made for the pasteurized one at 0 and 24 h. Some flavonoid molecules show a relatively high thermal stability and can undergo a less steep decrease of concentration compared to phenols (Chaaban et al., 2017; Elhamirad et al., 2012). The higher stability of some flavonoid molecules, paired with the disruptive action of the treatments may have produced a higher TFC compared to the one in the fresh sample. Also, a significant increase in TFC was recorded at 72 h of fermentation in all samples, with the exception of the FPT ones. Several studies have demonstrated how fermentation can increase both TFC and TPC in several matrices (Adetuyi et al., 2014; Liu et al., 2020), although we didn't see a raise in TPC, so these variations might be linked primarily to the type of food matrix being fermented.

Overall, it's possible to say that, differently from TFC, all parameters showed a decrease with the progression of fermentation, generally reaching a minimum at 48 h of fermentation. No literature is available on the specific relationship between phenols and flavonoids and pH, although it has been demonstrated how pH distresses may negatively affect the antioxidant properties of various matrices and molecules (Xie et al., 2018; Bayliak et al., 2016). When the pH stabilizes, generally after the 48 h of fermentation (Table 2), the negative effect of pH may be outbalanced by the enzymatic action of the microorganism that could free some bound molecules, thus increasing the TPC and TFC, together with the scavenging activities (DPPH, ABTS) (Adetuyi et al., 2014, Cömert et al., 2017).

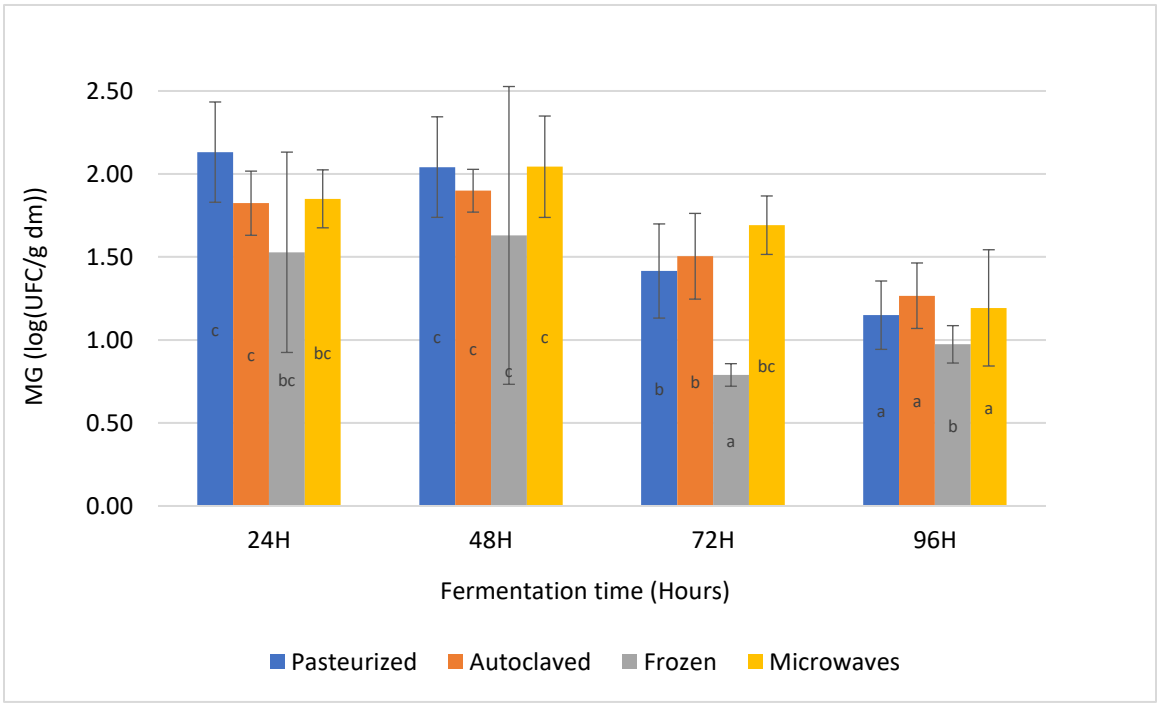
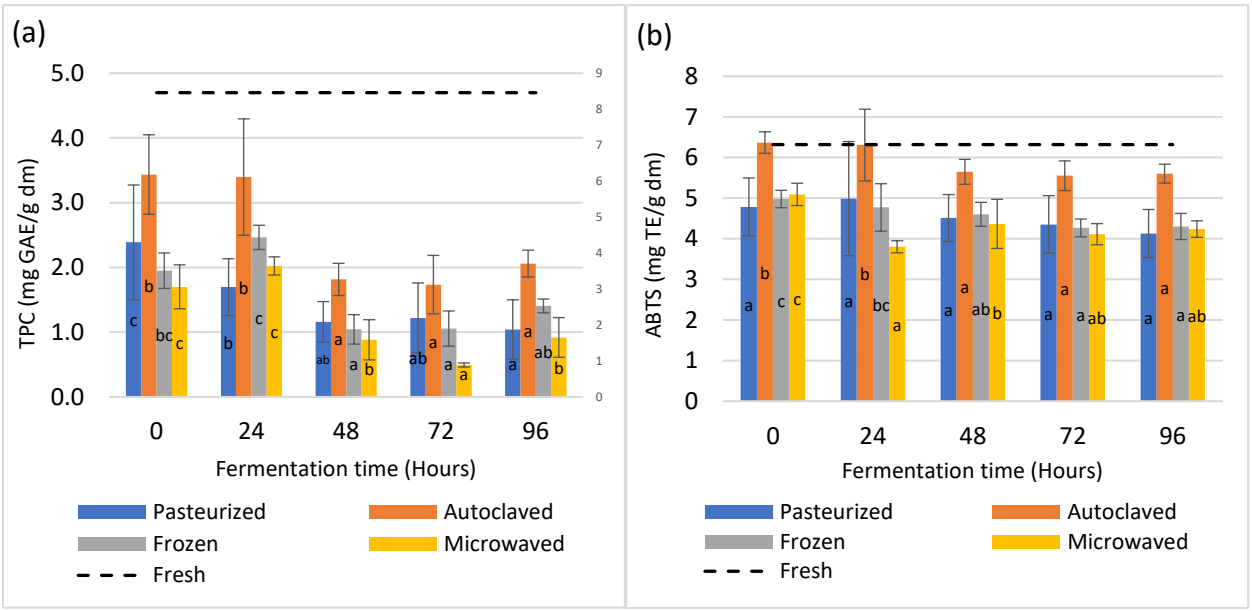


Figure 15: microbial growth expressed as absolute variation between microbial count after fermentation and microbial count on the freshly inoculated sample. Error is displayed as standard deviation. Different letters indicate statistically significant differences with a 95% confidence level (p-value<0.05) within the same series.



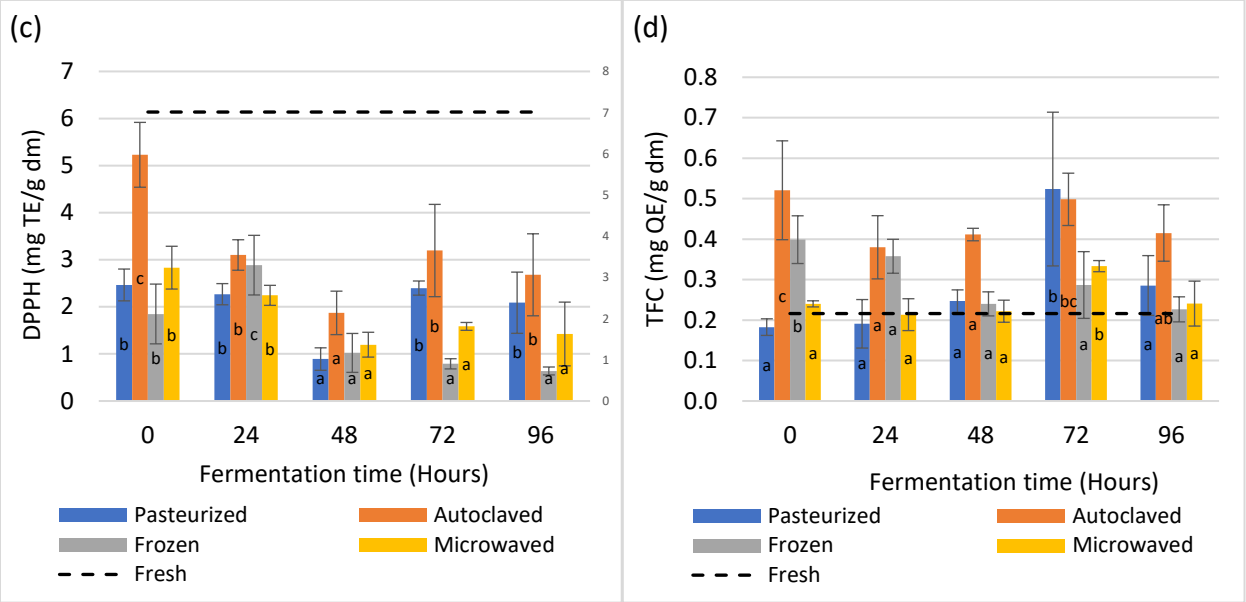


Figure 16: TPC, ABTS, DPPH and TFC variations are respectively represented by Fig.16a, Fig.16b, Fig.216c and Fig.16d. Error is displayed as standard deviation. Different letters indicate statistically significant differences with a 95% confidence level (p-value<0.05) within the same series.

Table 2: pH variation over the 96 h of fermentation with respect to the differ-rent thermos-physical pre-treatments applied to the broccoli stalks.

	Fermentation hours				
Pre-treatment	0	24	48	72	96
Pasteurized	$6.09_{c} \pm 0.02$	$3.91_{b} \pm 0.04$	$3.67_{a} \pm 0.02$	3.65 ± 0.03	3.64 ± 0.02
Autoclave	$5.9_{c} \pm 0$	$3.88_{b} \pm 0.02$	$3.71_{a} \pm 0.06$	$3.66_{a} \pm 0.02$	$3.67_{a} \pm 0.10$
Frozen	$6.15_{c} \pm 0.02$	$4.16_{b} \pm 0.06$	$3.71_{a} \pm 0.03$	$3.80_{a} \pm 0.18$	$3.71_{a} \pm 0.07$
Microwaved	$6.05_{d} \pm 0.02$	$3.84_{c} \pm 0.03$	$3.70_{b} \pm 0.02$	$3.64_{a} \pm 0.02$	$3.62_{a} \pm 0.03$
Different letter in the same row indicates statistically significant differences with a 95% confidence level (p-value<0.05)					

As expected, pH generally decreased with statistical significance down until the 48 h, due to the production of mainly lactic and acetic acid by bacterial activity (Corsetti and Valmorri, 2011), before remaining stable throughout the 72 and 96 h. The stabilization of pH is clearly connected to the reduction in microbial activity.

5. Conclusions

The results we obtained showed how none of the treatments that were imposed on the matrix had any positive effect on the content of antioxidants and antioxidant activity, exception made for flavonoid content. A less steep decrease has been observed in the autoclave pre-treatment case, which probably is more disruptive in regard to cell structures, liberating more bound antioxidative species and partially balancing the inactivation of said substances. Although, even in this case the net variation after the disruptive treatment is negative, meaning that applying such conditions is not convenient. This is even more evident considering how time and energy consuming is the application of a high pressure and high temperature treatment to a large volume of substrate. Fermentation doesn't seem to positively affect the content of antioxidants. One hypothesis might be that the pH decrease has a negative impact on the stability of antioxidative molecules, and when it stabilizes, the microbial action liberates some of the antioxidant species which have higher tolerance to low pH. This may cause the slight growth of all parameters registered between the 48 and 72 h.

Also, no significant difference was recorded in the microbial growth between different pre-treated samples. The variations that have been observed are most likely caused by random events and not connected with the chemical and physical changes imposed by the pre-treatments.

In regard to the chemical-physical changes, no parameter studied seems to be affected by the pre-treatments, with some exceptions between the different microwave combinations.

This has been demonstrated by the microbial growth, that didn't show particular variations between the microwave treatments, and also during the comparative study regarding the four different pre-treatments.

The little-to-no effect of the fermentation and pre-treatments may be explained by the low content of antioxidants in the stem, or the presence of particularly thermally unstable antioxidative molecules that are deeply affected by the thermal treatments, like pasteurization. One opportunity might be to avoid the rehydration of the matrix after microwaving. During the setting up of the experiments we observed how microwaves decreased the a_w of the sample due to water evaporation, but also concentrated the content of antioxidant species and fermentable sugars. The decrease in a_w may not be enough to affect the microbial growth of *Lactiplantibacillus plantarum*, which is able to grow pretty much at maximum potency in the range of 0.9-1 a_w (Bucio et al., 2005), while being enough to increase the availability of nutritional substances for the microbial population to grow and the concentration of bioactive compounds, such as antioxidants.

During our work, we considered pasteurization as a fundamental treatment to obtain the almost exclusive presence of the selected *Lactiplantibacillus plantarum* inoculum during fermentation. One interesting possibility would be to totally avoid any thermal treatment, limiting the imposition of selective conditions by chemical-physical means. For example, salt has been used as main selective agent since the first lactic fermentation products have been discovered, and it is still used nowadays for its important action on the microbiota of fermented products (Müller et al., 2018). The combination of sodium hypochlorite and an appropriate amount of salt, to be determined, may offer a sufficient control of the microbial population while having a less harsh impact on the content of antioxidants compared to the thermal treatment of pasteurization.

All this considered, the possibility of the reutilization of broccoli stalks remains very interesting and a good example of the re-valorization of food parts that would normally be considered as non-edible waste. The importance of fermentation applied to these studies shows how versatile and potent tool it can be to achieve beneficial products and to recover commonly non-eaten vegetable tissues.

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