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CICLO XXXIV

Fermentation as a strategy to produce high value-added compounds from by-products and waste

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Abstract

One-third of food produced worldwide is lost or wasted every year and this became an economic, ethical, and environmental problem. Waste and by-products are generated along the entire food supply chain and, as a result, have led the United Nations to introduce regulations to reduce losses. In this context, it is introduced the concept of circular economy that means reuse, repair, and recycling the existing materials and products, closing the loop and giving value to waste materials. There are several ways to obtain value-added compounds from by-products and it refers to the bio-refinery techniques which is emerging as a sustainable option in the scientific community. As food waste is rich in nutrients, it can support the growth of a variety of microorganisms, and several studies focused on the microbial fermentation of different by-products to obtain different value-added compounds like organic acids, aroma compounds, and antimicrobials. Among the variety of possible starters to conduct fermentation processes, lactic acid bacteria (LAB), with recognition as Qualified Presumption of Safety (QPS)-status by EFSA, are the most important microorganisms associated with fermentation and thus exploited for industrial bioprocesses. Recently, the exploitation of LAB strains to valorize by-products and waste into high value-added products has been described but their potential on bio-transformations is still an untapped biotechnology resource. Different metabolites of industrial interest, such as bioactive molecules, can be produced by lactic acid fermentation starting from low-cost substrates. Solid-state fermentation offers several advantages such as low cost, simple equipment, and most importantly, it permits the use of agricultural and agro-industrial by-products as substrates. The use of these materials as the culture medium of microorganisms represents a potentially significant reduction in the cost of bioprocesses and the conversion of the waste into products with commercial value-added. In this context, the PhD thesis was divided into two main activities: the first one is the screening of different lactic acid bacterial strains for the modification of aromatic profile, while the second one is the production of antimicrobial compounds during fermentation and the study of optimization models to evaluate their use in food industry.

Introduction

In recent years, we witnessed a rapid increase in food loss and waste. Indeed, it is estimated that one-third of food produced worldwide is lost or wasted every year and this reflects an economic loss about $1 trillion annually (Spang et al., 2019). This became also an ethical and environmental problem. Globally, almost two billion people have nutritional deficiencies and 800 million suffer from hunger (Blas et al., 2018). A recent study reveals that around 31% of food remains available but not consumed in the United States due to losses at the retail and consumer level. With a quarter of this amount, it would be possible to feed million hungry people in the world (FAO et al., 2018). Halving food loss and waste would also potentially increase the availability of agricultural production needed to meet demand in 2050 (Chen et al., 2020).

The environmental impact of food production is calculated on the basis as it requires many resources such as fuels, land, water, and raw materials but also at agricultural levels with fertilizer application or livestock farming, which produces a significant emission as methane derived from ruminants (Scherhaufer et al., 2018). As result, when food is wasted all the associated activities and emissions created in the food supply chain upstream are vain.

For all these reasons, this problem became one of the 17 Sustainable Development Goals (SDGs) of the United Nations. The ultimate goal is to halve per capita food waste at the retail and consumer level by 2030 and reduce food losses along with the food production and supply chain (United Nations, 2015).

In literature, there are many definitions for food waste and food loss that differs according to the different stage in which they are generated. In general, *Food loss* is defined as the decrease of food resulting from decisions and actions by food suppliers in the chain, excluding retail, foodservice, and consumers that are responsible for *Food waste* (Socas-Rodríguez et al., 2021). The term by-product, defined as a product resulting from the primary production of another product, fits into this context. By-products are for example peels and seeds of fruit and vegetables, husk of nuts, sugar beet, wheat bran, etc. Recent

studies indicate that the groups with the highest loss from post-harvest to distribution are roots, tubers (25%), fruit and vegetables (22%), followed by meat and animal products (12%), and finally cereals and pulses (9%) (Socas-Rodríguez et al., 2021).

These materials are often discarded because they are not part of primary production but conserve a nutritional value and are still rich in many bioactive compounds such as polyphenols, vitamins, proteins, or fats. As a result, this become an interesting topic for the scientific community in the last decades (Figure 1), that searching new strategies to better utilization of food waste and recovery bioactive compounds from food by-products. In literature are present different studies that range in different fields (Figure 2), from an economic and environmental point of view (Beretta & Hellweg, 2019; Chalak et al., 2016; Hassan et al., 2021; Kuiper & Cui, 2021), to the chemical and microbiological area (Chollakup et al., 2021; Feng et al., 2021; Freitas et al., 2021; Kim et al., 2022; Silva et al., 2020).

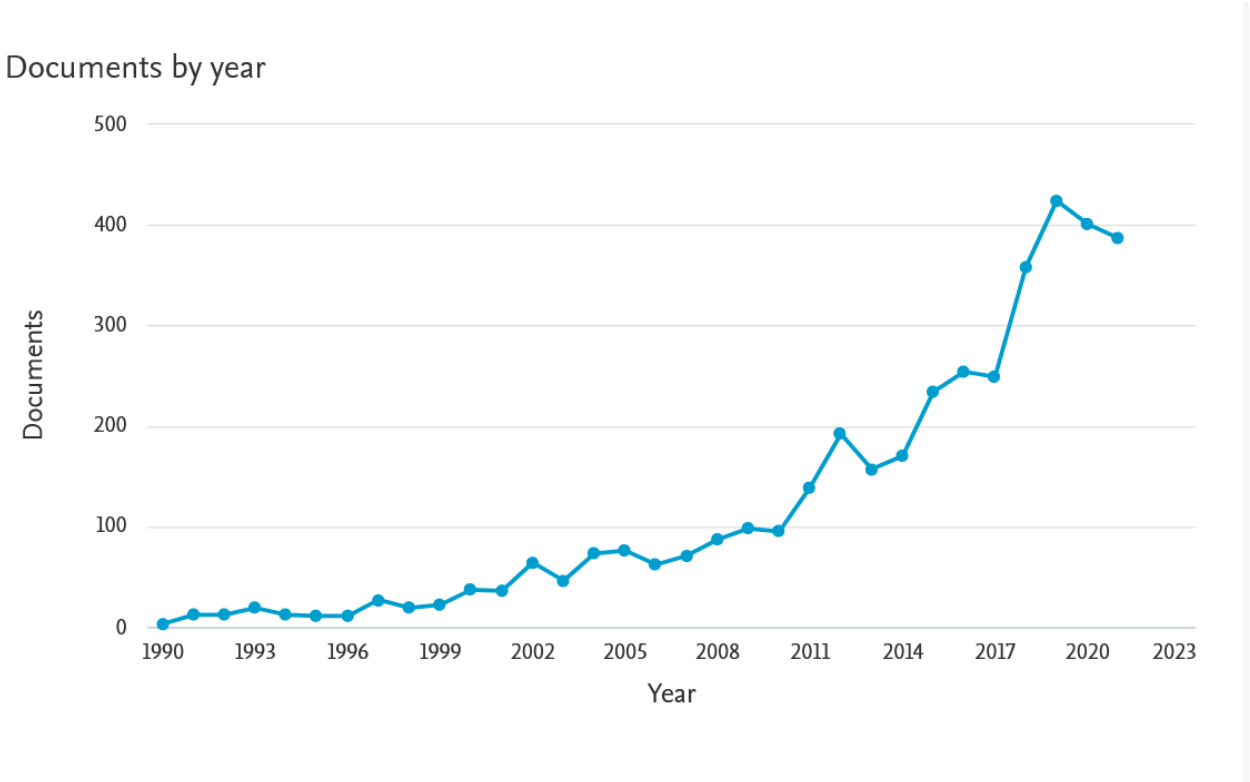

Figure 1 Numbers of papers published in the last thirty years about food by-products (Source: SCOPUS)

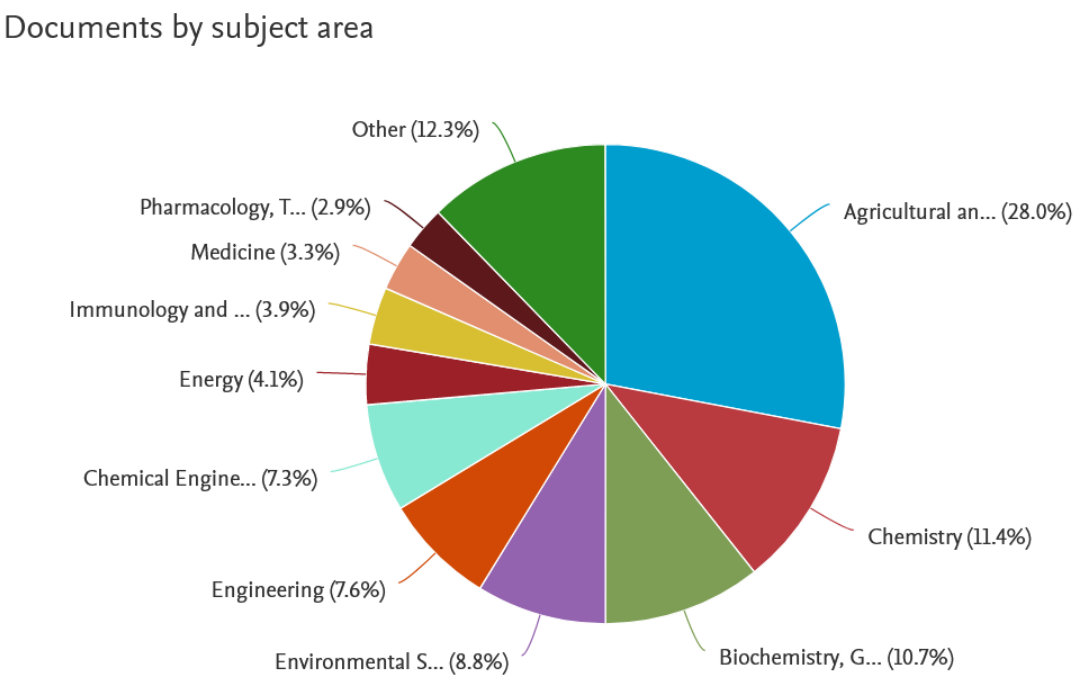

Figure 2 Research area of published papers in the last thirty years about food by-products (Source: SCOPUS)

Reducing the food waste generation and adding value to food by-product is part of the European Union's Circular Economy Strategy which aim is to increase resource by maintaining the value of materials through closing the loop of the product life cycle (EU Commision, 2014). Indeed, recently, the Member States of the European Union must adopt prevention measures to be included in waste management plans or environmental policy programs, according to Directive 2018/851/EU, to establish a system for quality control, traceability, waste calculation, and verification of waste prevention objectives (Zarbà et al., 2021).

A circular economy can be defined as an alternative to the current economic model based on a linear system of "produce, consume and discard" (Lehtokunnas et al., 2020). Indeed, the circular economy means to reuse, repair, and recycle the existing materials and products, closing the loop. In this perspective, what was previously considered as waste becomes a resource (Jurgilevich et al., 2016). In the food system, this principles implies reducing the amount of waste generated, re-use of by-products and food waste, and nutrient recycling.

These practices are fast catching up in the industries as they are facing serious problems in terms of management of resources, and the requirements to comply with governmental regulations in terms of disposal (Chandrasekaran, 2012). As a result, food by-products cannot be neglected since their valorization of them lead to generating several value-added products of commercial significance.

Reducing waste generation in the food industry is possible through efficient material use, process improvement, and lower operational costs. Furthermore, precise forecasting, accurate supply, and demand prevent waste overproduction (de Moraes et al., 2020).

Recycling food waste to obtain commercial products and energy is known as a bio-refinery concept and is emerging as a sustainable option in the scientific community. The bio-refinery as a tool for the management of food waste is considered environmentally friendly due to less greenhouse gas emissions, and reduction in the environmental burden of their disposal. Several products are obtained depending on the techniques used. For instance, renewable bioenergy and biofuels are generated through anaerobic or aerobic digestion, and microbial fermentation processes with minimum emission and greater yields (Nayak & Bhushan, 2019).

As food waste is rich in nutrients, it can support the growth of a variety of microorganisms, indeed in the last years, several studies focused on the microbial fermentation of different by-products using both bacteria, yeast, and fungi to obtain different value-added compounds like organic acids, aroma compounds, antimicrobials. Generally, fermentation is defined as a metabolic process in which organic substrates are oxidized to liberate energy. Is one of the oldest techniques applied in food preservation and processing, which leads to products with unique flavors and texture characteristics appreciated by consumers (Rollán et al., 2019). There are different strategies to conduct fermentation processes and one is solid-state fermentation (SSF) in which microorganisms are cultivated in close contact with the solid substrate and are gain attention as it requires low energy and water consumption. The results of fermentation process are determined by the substrates used for the growth of microorganisms, the type of starter, and the process conditions. Among the variety of possible starters to conduct fermentation processes, lactic acid bacteria are common

bacterial species found in dairy, meat, and vegetable niches, as well as the gastrointestinal and urogenital tracts of humans and animals, soil, and water. They are well known for producing lactic acid as the principal end product of their anaerobic metabolism, as well as leading to a range of metabolites that have significant effects on the nutritional, sensory, and technical properties of fermented foods. Moreover, they obtained the recognition as GRAS (Generally Recognized As Safe) by the US Food and Drug Authority and included in the QPS (qualified presumption of safety) list by the European Food Safety Authority (EFSA, 2016). As a result, LABs have been widely employed as starter cultures, as probiotics, and in the creation of novel chemicals compounds (Ruiz Rodríguez et al., 2019).

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Aim of the thesis

This PhD research project aims to apply steered fermentation processes on waste materials to obtain high value-added products, in particular aroma compounds and antimicrobials. The first part of the research was focused on the exploitation of strains of lactic acid bacteria biodiversity, exploration of different by-products, and the evaluation of aromatic profiles after fermentation. The second part of the project involved the study of antimicrobial compounds obtained from fermentation and the evaluation of the possible use in commercial products using experimental optimization designs.

- **Chapter one** offer a general overview of the potential use of lactic acid bacteria as starter for fermentation of waste and by-products to obtain volatile compounds

- **Chapter two** Focused on the ability of microbial strains to convert by-products with different composition

- **Chapter three** discuss the antimicrobial activity of insects and their importance. A case study on the effect of fermentation on insect waste is illustrated.

- **Chapter four** investigate the use of antimicrobial compounds derived from the fermentation of tomato by-product, against spore-forming bacteria

Chapter 1

Fermentation of Agri-Food Waste: A Promising Route for the Production of Aroma Compounds

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Abstract

Food wastes and by-products are generated along the entire food transformation and storage chain. The large amount of waste deriving from the whole process represents not only a great economical loss, but also an important ethical and environmental issue in terms of failure to recycle potentially reusable materials. New clear strategies are needed to limit the amount of waste produced and, at the same time, promote its enhancement for further conversion and applications in different industrial fields. This review aims to give an overview of the biological approaches used so far to exploit agri-food wastes and by-products. Application of solid-state fermentation by different microorganisms (fungi, yeasts, bacteria) to produce several value-added products was analyzed, focusing on the exploitation of lactic acid bacteria as workhorses for the production of flavoring compounds.

Are We Sure They Are Really Waste?

In the last decades, a new challenge concerning the reduction of food waste (FW) and food loss has been raised for the world population. Indeed food production is increasing every year worldwide, and the Food and Agriculture Organization of the United Nations (FAO) has estimated that one-third of the products intended for human consumption (corresponding approximately to 1.3 billion tons/year) is wasted or lost every year (FAO, 2013).

This event, in the developing countries, is mainly originated in the first steps of the food supply chain, due to the technical and management limits in post-harvest, processing and distribution steps. On the other hand, in medium and high-income countries, food is lost or discarded in the final steps, by retailers and consumers, due to the high productivity and quality standard required by the market (FAO, 2015). As a result, many resources are lost in terms of land, water and nutrients, and therefore production, processing, manufacturing and transports become a waste of energy.

Considering the growth of the global population up to 2050, to ensure appropriate food supply, food production must increase in the next decades (United Nations, 2015), causing a rise in FW, ultimately posing a serious problem in terms of waste management and disposal.

Thus, the modern challenge is to minimize FWs, optimize production processes and move from a linear consumption and production model to a new one organized according to a circular economic strategy. In this view, FWs are considered as by-products and resources, and several studies about wastes and by-products valorization have been conducted in the last decades (Barba et al., 2017; Clementz et al., 2019; Gullón et al., 2020).

FW is meant as any part of food that is discarded regardless of its potential con-tent of compounds retaining a high value (Hartikainen et al., 2018). Depending on its origin of production, FW can be characterized by a variable chemical composition of carbohydrates, proteins, lipids, and other components that could be used for different industries and fields (Galanakis, 2020) such as biorefineries, biomaterial, pharmacy, cosmetics and aroma

industries. Using food by-products could be advantageous for the aroma industry due to market demand for natural products and for a low material cost.

This review aims to provide insights into the strategies performed to exploit waste and by-products from the agri-food chain. In particular, this work is focused on the application of Solid-State Fermentation (SSF) to produce several value-added products, such as flavor and aroma compounds. To better delineate a specific topic, considering the metabolic potential of lactic acid bacteria (LAB), we decided to present a detailed overview of their employment in the production of natural flavors.

Agri-food Waste: A Rising Problem or a Valuable Resource?

Among the different food sectors, it is estimated that fruit and vegetables represent a large part of waste production, notably in detail about 45% of the total produced amount is lost in the production and consumption chains, generating a great amount of waste materials (Fidelis et al., 2019; Sagar et al., 2018). Wastes and by-products can be classified into four source groups, according to the step in which they are generated along the entire agri-food chain: (i) in the fields, before harvesting, due to pest infestation and crop damaged by unfavorable weather conditions; (ii) in post-harvest and transport, where spoiled and bruised fruit and vegetables are discarded; (iii) in the different manufacturing steps process such as peeling, washing and slicing; (iv) in retail and market, due to natural spoilage at the end of shelf life (Ravindran & Jaiswal, 2016).

Still, the recovery and management of these wastes are not trivial. Seasonality, distribution over the territory, perishable nature due to the high content of water and nutrients and the heterogeneity of the product, may represent possible difficulties and problems for agri-food wastes management (Girotto et al., 2015).

A feasible and economically sustainable agri-food wastes recycling requires large volumes of raw materials concentrated in the same area, a high degree of homogeneity, and a careful analysis of downstream costs (Pfaltzgraff et al., 2013). In keeping with this, an industrial symbiosis could be a productive and useful strategy. The FWs generated by diverse companies could be transferred to other industries, that could use and transform them for other purposes, in a circular economy perspective (Mirabella et al., 2014).

In the last decades, the fate of organic wastes was different according to different types of areas. In rural areas and farms, organic wastes were commonly used as livestock feed or were composted into humic substances used as fertilizer for fields. Instead in urban areas, household wastes, with a more complex composition, were destined for incineration or landfilling thus posing significant environmental problems regarding air pollution and groundwater contamination (Sánchez et al., 2015).

The theory of waste valorization is strictly associated with sustainable technologies for recycling and reuse. The concept behind waste valorization is enhancing the value of a product, converting wastes into other resources with an added value. The possible resulting products could be new chemicals, materials, fuels, and energy, just like a lot of other products advantageous for a local/global economy.

Furthermore, the valorization and recycling potential of agri-food wastes and by-products can assure sustainable food production and at the same time guarantee food security. Interestingly, some materials deriving from food industry can be reused thanks to their distinctive properties, exploiting their physic-chemical characteristics, in many different industrial sectors. Lignocellulosic by-products like soy stalk, corn stalk, wheat straw, could be used as reinforcement into biodegradable polymer matrices to prepare building products with high strength or in the paper industry (Bhuyan et al., 2020).

With this in mind, currently the most promising frontier seems to be biorefineries. Biorefineries are industries that starting from biomass feedstock and through extraction processes, chemical and biological reactions, can recover the nutrients to create value-added products, and green energy (Carmona-Cabello et al., 2018).

In these new industrial realities, the organic materials can be treated by various techniques such as SSF, Submerged Fermentation (SmF), Anaerobic Digestion (AD). For example, chemical compounds like bioethanol and biobutanol are obtained from starchy and lignocellulosic by-products through fermentation by *Saccharomyces cerevisiae* and *Clostridium acetobutyricum* respectively. In this case, saccharides must be enzymatically pre-treated to break down the polymer chains into glucose monomers, subsequently metabolized by microorganisms (Girotto et al., 2015). Different types of biofuels can be

obtained depending on diverse starting substrates. For example, from the transesterification of vegetable oils and animal fats it is obtained biodiesel, whereas methane and biohydrogen are produced both by AD of any biodegradable substrate and by pyrolysis (Carmona-Cabello et al., 2018). Another source of by-products for bioethanol production is the sugarcane industry, whose by-products were proposed for AD, microbial fermentation, and microalgae cultivation (Sydney et al., 2021).

With production of over 700 Mt/year, rice, wheat, and corn crops represent one of the main food sources worldwide. After their processing and milling, huge volumes of by-products remain like stalks, straws, husks (Bledzki et al., 2010), and due to high cellulose and hemicellulose content could be used for biofuels production.

In recent years, food and beverage companies are paying great attention to bio-materials coming from renewable sources, being biodegradable and compostable, in a perspective of environmental protection. Afterward, those are only few examples related to new materials obtained from agri-food wastes. Bioplastic materials can be produced by lactic acid fermentation of agro-industrial residues and household wastes through engineered microorganisms. Polylactate (PLA), deriving from the polymerization of lactic acid monomers, is mainly used for the manufacturing of compostable products with short shelf-life, such as films and bags for packaging, thanks to its good transparency, biocompatibility, and processability (Armentano et al., 2013). Similarly, polyhydroxyalkanoates (PHAs), with the most representative poly[(R)-3-hydroxybutyrate] (PHB), are used in different applications including packaging (Masood et al., 2015).

Many compounds are also extracted and used as additives, colorants, or ingredients in food. Among them are pectin from citrus and passion fruit peels, apple and peach pomace, which are used as gelling agent, thickener and food stabilizer (Naqash et al., 2017); carotenoids from tomato skin and pomace (mainly lycopene and β-carotene), carrot by-products, mango, and orange peel. Dietary fiber can be recovered from banana peels and used in baking (Galanakis, 2020). Orange juice fibers by-products have been characterized highlighting a potential use as fat replacer in ice cream (de Moraes Crizel et al., 2013); finally, anthocyanins, could be extracted from berry by-products, grape pomace and several exotic

fruits (Klavins et al., 2018). In particular, bioactive compounds like polyphenols, flavonoids and minerals are extracted from the losses and by-products of fruit and vegetable industries and find use in the production of functional food and nutraceutical sector (Ben-Othman et al., 2020).

This recycling and reuse policy require not only technical knowledge but also a change of global mindset. Wastes and by-products are often seen as useless stuff to throw away, without thinking about their possible reuse. Furthermore, it is necessary that these wastes could be seen from a wider perspective than just one company or sector. To reach the waste recycling goal, the industries must communicate with each other, to establish a close system of valorization, where an industrial symbiosis between diverse sectors can be a productive and useful strategy.

Repurposing Agri-food Waste by Solid-State Fermentation for the Production of Aroma Compounds

Fermentation is a well-known technique, dating back to ancient civilities for the production of food commodities such as bread, wine, and fermented milk. The microbial transformation made the products particularly appreciated for their easier digestibility, flavor, and longer shelf life. Nowadays, there is a greatest interest in improving health, nutritional, technological and organoleptic qualities in fermented foods and, thanks to the development of new starter cultures, it is possible to guide microorganisms on different substrates for the production of new compounds including flavors (Pereira et al., 2020). The biodiversity of microorganisms can be exploited not only to produce foods with peculiar and appreciated aromatic notes, but also to convert diverse precursors into fine biochemicals, in a view of biocatalysis, such as aroma compounds and fragrances.

Fermentation is the process operated by microorganisms to break down organic compounds to obtain energy, through anaerobic metabolism. This biological process is characterized by low costs, low energy consumption and low wastewater generation, and can be exploited for the repurpose of organic wastes into value-added products (Tlais et al., 2020). In SSF, fungi, yeasts and bacteria grow on the surface of various organic sub-stances, which act as physical support for their development without adding water (Yazid et al., 2017). Fungi and

yeasts are the microorganisms of choice for this application, which is conducted at a moisture content between 40-80% (Ali & Zulkali, 2011). Alternatively, SmF can be applied, with a moisture content of about 80-95%.

Each technique has both advantages and limitations: SmF is routinely operated for the production of a variety of products, ranging from beverages such as wine and beer to more sophisticated methods for the cultivation of animal and plant cell cultures for biomedical application. Despite its broad range of applications, SmF has some drawbacks regarding process scale-up, due to the requirement of large volumes of water compared to low yield (Ben Akacha & Gargouri, 2015). SSF, on the other hand, is traditionally applied in the manufacturing of various Asian fermented foods and has recently gained attention due to low operating costs, reduced water consumption, and the lack of requirement for sophisticated bioreactors. However, the drawback of this technique is limited control of the environment within the bioreactor, accompanied by high costs for end-product recovery, and downstream processing (Arora et al., 2018). The choice of the most suitable media and microorganisms in sight of optimization and planning of downstream processing steps, as well as the possibility of using low- to zero-cost substrates, such as food waste, represent the key to success for this technique (Singhania et al., 2009).

Indeed, several added-value products can be recovered from agri-food waste substrates after fermentation, such as antibiotics, pigments, biosurfactants, hydrolytic enzymes, plastics, pesticides, bioactive compounds, as described in different studies (Cerda et al., 2019; Couto & Sanromán, 2006; Lizardi-Jiménez & Hernández-Martínez, 2017; Martins et al., 2011; Yazid et al., 2017). Several agro-industrial wastes can be used as immobilization carrier in SSF, as reported by Orzua et al. (2009), and represent an opportunity for the synthesis of industrially relevant metabolites.

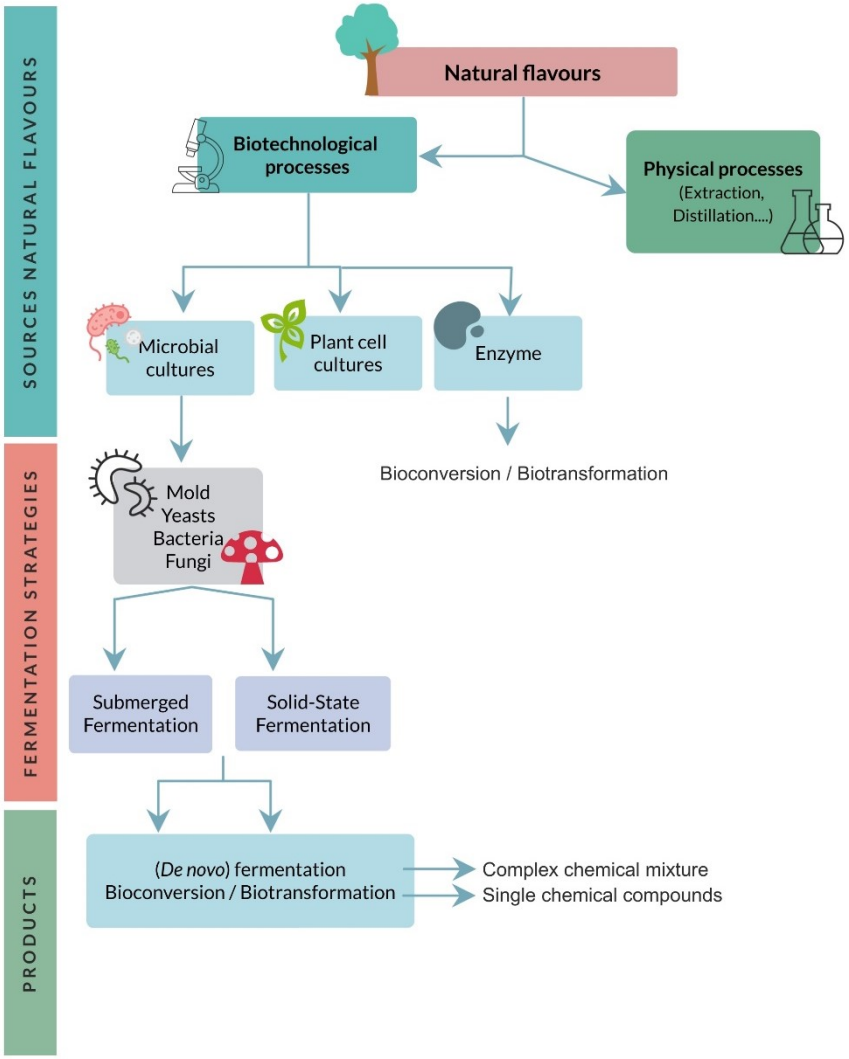
Aroma compounds production is a promising field for the application of SSF. Flavor compounds can be chemically synthesized, extracted directly from natural matrix, or derived from biotechnological processes (Sales et al., 2018). These approaches offer the possibility of obtaining additives suitable for various industrial applications, which can be

labeled as “natural” and environmentally friendly as they need less solvents for extraction when compared to chemical synthesis methods (Felipe et al., 2017).

Currently available biotechnological processes for aroma production (Fig. 1) make use of enzymes, microbial cultures, or less frequently, plant cell cultures (Longo & Sanromán, 2006).

The application of enzymes for the production of aroma compounds is based on their addition to the substrate during the flavor production process. Enzymes like lipase, protease, glucosidase, cellulase, etc. can act on specific precursors of aromatic compounds. The result is a bioconversion of organic material into an aromatic product, through a single or multistep catalyzed reaction (Ben Akacha & Gargouri, 2015). One of the most promising applications is the use of lipolytic enzymes to produce esters (Kumar et al., 2020) even if the scale-up of the application on an industrial scale is still complicated due to the high cost. Pre-treatment with lipolytic enzymes of grease waste before SSF was also proposed as an effective strategy to recover fatty acids from degraded grease waste (Kumari et al., 2017).

Figure 1. Processes to get natural flavors



When we describe the effect of a microbial culture on a substrate, we can distinguish between bioconversion/biotransformation and fermentation. In biotransformation, the microorganism converts a precursor into a product of interest, by a single or multistep reaction such as the conversion of ferulic acid into vanillin and stereo or regioselective changes of terpenes, as reviewed by Sales et al. (2018). The biotransformation process is particularly promising for the use of engineered microorganisms. Starting from the enzyme which catalyzes the reaction, and knowing how this enzyme works, it is possible to insert the gene coding for that enzyme into the genome of high-producer and better adaptable microorganisms, to increase the effectiveness and efficiency of the process. The gene coding for the lipoxygenase of *Pleurotus sapidus* has been cloned, for example, in *Escherichia coli* thus enabling it to convert valencene into the grapefruit flavor nootkatone (Zelena et al., 2012).

Cereal bran and other agricultural wastes such as sugar beet pulp, rice bran oil, palm oil biomass, pineapple by-products have been studied as sources of ferulic acid, a precursor for the conversion of bio-vanillin with natural or engineered bacteria (Tang & Hassan, 2020; Zamzuri & Abd-Aziz, 2013).

Biotransformation is an easier process to be applied on an industrial scale since it leads to the production of single aroma compounds, but it is difficult to carry out using waste (Carroll et al., 2016). Furthermore, the use of genetically modified organisms is not perceived as truly "natural" by consumers (Boccia et al., 2018).

In fermentation (*de novo* synthesis) an entire metabolic pathway is involved. The catabolism of carbohydrates, proteins and lipids contributes to the production of primary metabolites, which are subsequently converted into a mixture of aromatic compounds (Sales et al., 2018). When agri-food wastes or by-products are used as a substrate, glucose supplementation is often required mainly to support the initial growth of microorganisms, despite high concentrations that might lead to catabolite repression phenomena (Soares et al., 2000). An interesting strategy to overcome nutrient limitations occurring in SSF on agri-food waste is mixing different waste substrates, developing fermentation substrates that do not require nutrient supplementation. With this aim, a SSF of mixed agri-food wastes with *Kluyveromyces marxianus*, *S. cerevisiae* or an undefined mixed culture from kefir, shown to be a promising approach for the development of biorefineries aimed at the production of biomasses and volatile aroma compounds (Aggelopoulos et al., 2014).

The production of specific compounds has been demonstrated to be inducible by adding precursors; for example, it has been consolidated that the addition of leucine and valine to the growth substrates, including agri-food waste, leads to the formation of isoamyl acetate with a strong banana aroma (Bramorski et al., 1998; Quilter et al., 2003), due to the Ehrlich pathway that leads the catabolism of the amino acid and production of esters as final products (Shetty & Sarkar, 2020). Other derivatives of the Ehrlich pathways are the rose-scented volatile compounds 2-phenylethanol and 2-phenethyl acetate, which were synthetized through SSF of sugarcane bagasse upon addition of L-phenylalanine as a precursor, from the yeasts *K. marxianus* and *Pichia kudriavzevii* (Martínez et al., 2017, 2018a).

Several studies have been conducted on the use of microbial cultures (especially molds and yeasts) growing on agri-food wastes and by-products, to produce aromatic compounds, and a list of the main results is reported in Table 1.

Table 1. Aroma production by SSF of agri-food wastes/by-products, using molds and yeasts.

	Agri-food Waste	Pretreatment(s)	Aroma	Reference
Mold				
Ceratocystis fimbriata	Citrus pulp + 25 % sugarcane molasses (+ 50% soya bran)	Drying, milling, sieving	Fruity aroma	(Rossi et al., 2009)
	Coffee husks (+glucose)	Milling, steam treatment	Pineapple aroma (acetaldehyde, ethanol, isopropanol, ethyl acetate)	(Soares et al., 2000)
	Coffee husks	Drying, milling, sieving, sterilization	Fruity flavor	(Medeiros et al., 2006)
Rhizopus oryzae	Cassava bagasse, apple pomace, amaranth, soybean	Drying, milling, sieving, sterilization	Fruity aroma (+ amaranth and + banana aroma)	(Bramorski et al., 1998)
	Wheat bran, cassava bagasse, sugarcane bagasse	Milling, sieving, sterilization. For sugar cane bagasse: preliminary washing	Fruity aroma (strong banana aroma)	(Christen et al., 1997)
	Cassava bagasse, apple pomace, soybean, amaranth, soybean oil	Grinding, drying, sterilization	Acetaldehyde, Ethanol, 1-Propanol, Ethyl acetate, Ethyl propionate, 3-Methyl butanol	(Christen et al., 2000)
Trichoderma viride	Sugarcane bagasse	N.d.	Coconut aroma, 6-pentyl-α-pyrone	(De Aráujo et al., 2002)
		Drying, milling	Coconut aroma, 6-pentyl-α-pyrone, from δ-Octalactone to Dodecalactone	(Fadel et al., 2015)
Trichoderma harzianum	Sugarcane bagasse	Drying, milling	6-Pentyl-α-pyrone	(da Penha et al., 2012; Ladeira et al., 2010)
Kluyveromyces marxianus	Apple pomace, cassava bagasse, sugar cane bagasse, sunflower seeds, giant palm	Drying, milling, sieving, sterilization	Ethanol, ethyl acetate	(Medeiros et al., 2000)
	Sugarcane bagasse + sugar beet molasses	Drying, milling, pH adjustment	Fruity aroma (43% alcohol, 35% esters)	(Martínez et al., 2017)
		Drying, milling, pH adjustment	Fruity aroma	(Martínez et al., 2018b)
Aspergilius niger, Penicilium cinnabarium	Rice brain oil residue (+ferulic acid)	Water-ethanol extraction, pH adjustment, filter sterilization	Vanillin	(Zheng et al., 2007)
Hanseniaspora velbyensis and uvarum, Saccharomyces cerevisiae	Apple peels	Drying, homogenization	132 volatile compounds	(Rodríguez Madrera et al., 2015)
Yeasts				

Pichia kudriavzevii	Sugarcane bagasse + l-phenylalanine	Drying, milling, pH adjustment	Rose aroma	(Martínez-Avila et al., 2020)
Saccharomyces cerevisiae	Citrus peels	Slicing, grinding	Isoamyl acetate, ethyl dodecanoate, ethyl decanoate, ethyl hexanoate	(Mantzouridou et al., 2015)
Yarrowia lipolytica (engineered)	Fatty feedstock	N.d.	Coconut like flavor (γ-dodecalactone, δ-decalactone)	(Marella et al., 2019)
Kefir (symbiotic yeasts and bacteria)	Food industrial wastes (cheese whey, molasses, brewer's spent grains, malt spent rootlets, orange and potato pulp)	Blending	Ɛ-pinene	(Aggelopoulos et al., 2014)

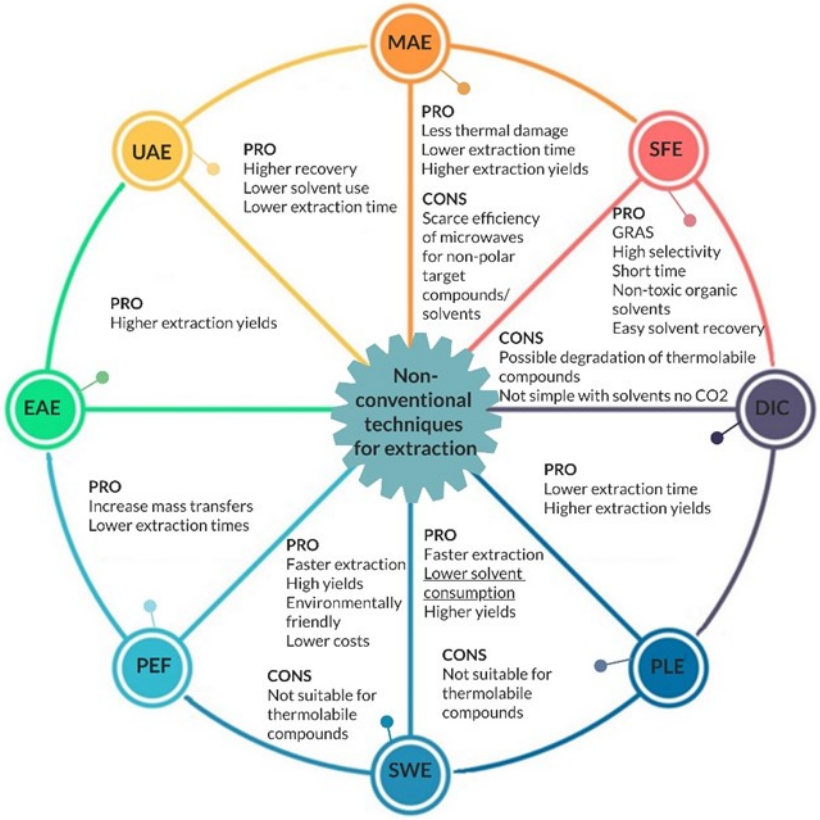
Optimization of SSF approaches relies consistently on the isolation of novel strain/substrate combinations, also due to the strain-specific capability of volatile compounds production, making strain selection a key point of the process (Rodríguez Madrera et al., 2015). It is also known that the microbial growth phase can affect the production of volatile compounds (Ricci et al., 2018; Willrodt et al., 2015), and optimization of SSF processes suggests that the metabolic state of the microbial cells can influence their synthesis (Martínez-Avila et al., 2019). Yet, the limited knowledge of bacterial physiology during SSF and regulation of pathways involved in aroma formation represents a limit in the optimization of strain selection and operating procedures (Xie et al., 2018).

The fermentation processes are followed by bioseparation processes such as ex-traction, purification, and recovery of the compound of interest. It is the most difficult step particularly for flavor components due to their volatility and low solubility (Sharma et al., 2020). It is necessary a deep knowledge of the properties of the target compounds and the matrix in which they are dispersed, to choose the most appropriate extraction method, as well as increase the selectivity and efficiency and thus obtain the maximum recovery of the product from SSF.

There are many techniques available for the isolation of flavor compounds, and although there is no correct technique in general, the challenge is to find the most suitable one capable of extracting the desired flavors in the best way, avoiding losses of volatile compounds during the process, due to aeration of SSF bioreactors (Try et al., 2018). Combination of different extraction and separation techniques (hybrid processes) often proves beneficial for

large-scale applications (Berger, 2007). Next to the traditional extraction methods, the development of non-conventional techniques aims to improve the efficiency of extractive processes of bioactive compounds. These techniques can reduce the time and temperature of extraction, maintaining high selectivity and high yield, using less dangerous solvents and that is why they are considered "green techniques" (Azmir et al., 2013). Considering all these aspects, it would be desirable that an increasing number of studies report preliminary cost analysis, to provide orientation for future development of industrial processes (Aggelopoulos et al., 2014). In Figure 2 are summarized these techniques highlighting the pros and cons of each.

Figure 2. Operating principles of non-conventional techniques with the main advantages (PRO) and disadvantages (CONS) compared to the traditional techniques. UAE ultrasound-assisted ex-traction; MAE microwave-assisted extraction; SFE supercritical fluid extraction; DIC instant con-trolled pressure drop-assisted extraction; PLE pressurized liquid extraction; SWE subcritical water extraction; PEF pulsed electric field; EAE enzyme assisted extraction.



Lactic acid bacteria: biological resources for volatile compounds production

LABs are widely used in food industry as starters, to drive the fermentation processes, and as probiotic sources. Their employment is easy also because they are recognized as GRAS (Generally Regarded As Safe), have a simple metabolism, can grow on many different carbon sources and have a good tolerance to environmental stresses such as pH and temperature (Mazzoli et al., 2014). In recent years the development of genome sequencing techniques and genetic tools has widened the range of applications, helping to control the bioproduction of value-added products and standardize the process. All these characteristics make it possible to exploit LAB in biorefineries, for the production of various types of value-added products such as: lactic acid, plastic polymers, ethanol, exopolysaccharides (with thickener and prebiotics function), antimicrobial molecules, food aromas and sweeteners (sorbitol, mannitol, l-alanine) (Mazzoli et al., 2014).

Recently, various studies have been dedicated to the lactic acid fermentation of different substrates (Ricci et al., 2019; Spaggiari et al., 2020) using *Lacticaseibacillus rhamnosus* for the evaluation of the aromatic component which undergoes modifications during the process. Fermentation can be used to increase the aromatic notes (Ricci et al., 2020) or to reduce the off-flavor components in the products (Martelli et al., 2020). The changes of the aromatic profile derived from the metabolism of bacteria which, depending on the various nutritional compounds available, produce different metabolites. Microorganisms metabolize the carbon sources for growth and energy production during fermentation. The resulting metabolites can be both aromatic compounds and aroma precursors (Shetty & Sarkar, 2020). These compounds have specific sensory attributes, for example, esters are characterized for the most part by sweet smell (Ben Akacha & Gargouri, 2015), while aldehydes usually bring floral or fruity notes (Sharmila et al., 2020).

Important and complex aromatic molecules are known to be generated by LAB during the fermentation of dairy foods. These compounds are synthetized as a result of primary metabolism of carbon sources (lactic acid and mixed acid fermentation), or by proteolysis and other secondary metabolism that occur during the cheese ripening step. Buttery flavor seems to be the most interesting compound produced by LAB in qualitative and

quantitative terms and is widely used in bakeries. The molecule that best expresses this flavor is diacetyl, followed by acetoin, butanediol and acetaldehyde. The above and other metabolites, such as ethanol and acetate, derive from pyruvate, giving a typical flavor to fermented foods. Only some species of LAB, with the ability to metabolize citrate, can produce diacetyl (Smid & Kleerebezem, 2014). The microbial synthesis of diacetyl, in *Lactococcus lactis*, is stimulated in acidic conditions and seems to be produced to control intracellular pH (García-Quintáns et al., 2008). Papagianni (2012) has examined several approaches that have been adopted to improve the production of diacetyl. In aerobic conditions the pyruvate pathway is shifted to the synthesis of α-acetolactate, the reaction is catalyzed by two enzymes: α-acetolactate synthase (ALS) or acetohydroxy acid synthase (ILVBN). Acetolactate can be either converted into diacetyl by an oxidative reaction or into acetoin by decarboxylation with the enzyme α-acetolactate decarboxylase (ALDB). Genetic engineering has attempted to inactivate the gene that expresses ALDB and overexpress the ALS and ILVBN genes, with little success. In contrast, the overproduction of NADH oxidase (NOX) in *Lactococcus lactis*, in addition to the inactivation of ALDB gene, has been shown to be successful in driving the metabolism of pyruvate in the production of diacetyl, rather than lactate (Hugenholtz et al., 2000). In this way, *L. lactis* has increased the diacetyl production and at the same time, it has reduced lactate production (Guo et al., 2012).

In this context, many efforts have been aimed at the construction of recombinant strains by metabolic engineering to enhance flavor production, as well as to screening of LAB collections, in particular *Lactobacillus* spp., to uncover the metabolic potential for the synthesis of flavor compounds and a better definition of acetoin production transcriptional activation (Bancalari et al., 2020; Flahaut & de Vos, 2015; Levante et al., 2020).

In recent studies, lactic acid fermentation was selected as a process for enhancing the flavor profile of fruit juice. Although most volatile compounds occur naturally in plants, they can be synthesized by microorganisms as secondary metabolites. Chen et al. (2019) observed a positive modification on aromatic profile of apple juice fermented with different LAB strains (belonging to the genus *Lactobacillus*), in particular an increase of alcohols like 2-ethyl hexanol and ethyl acetate with floral and fruity notes. Same results were recorded in other

fruit juices such as pomegranate (Di Cagno et al., 2017) where the fermented juice, with *L. plantarum*, had a more intense fruity note that can result from an increase in alcohols, ketones, terpenes, or in elderberry juice (Ricci et al., 2018), where LAB increased the volatile compounds typical of elderberry, like β-damascenone and various alcohols such as hexanol, 3-hexen-1-ol (Z), 2-hexen-1-ol (E), ethanol, 2-phenylmethanol, 2-phenylethanol and isoamyl alcohol, hexanol, 3-hexen-1-ol (Z) and 2-hexen-1-ol (E). During the fermentation of barley malt worth beverages (Nsogning Dongmo et al., 2017) with different LAB strains, it was recorded a higher aroma yield and fruity flavor due to an increase of some compounds like β-damascenone, furaneol, 2-phenylethanol and ethyl 2-methylbutanoate.

Activation of the metabolic pathways that lead to the formation of certain aromatic compounds is however closely related to the substrates used for fermentation and to the strain. Thanks to the unique portfolio of enzymes that LAB possess, they activate different metabolisms, like catabolism of aldehydes, synthesis and hydrolysis of esters, degradation of phenolic acids, lipolysis, proteolysis and peptidolysis (Szutowska, 2020).

Despite the number of studies on lactic acid fermentation showing an improvement in the sensory qualities in fermented products, the use of LAB for the production of aromas from waste and by-products has been little studied (Ricci et al., 2019; Spaggiari et al., 2020). LAB can grow on many different substrates, among which lignocellulosic by-products, agri-food and municipal solid wastes (Ali Abdel-Rahman et al., 2011; Wang et al., 2017).

Almost all LAB are not able to directly ferment complex polysaccharides like starch or hemicellulose, and therefore hydrolysis pretreatments (with related costs) are necessary, together with additions of amino acids, nucleotides, and vitamins (Mazzoli et al., 2014). For the direct exploitation of these economic feedstocks, some strategies have been implemented, including the co-cultivation of LAB with native cellulolytic microorganisms and the modifications of the gene pool by inserting hydrolytic genes for saccharification (Tarraran & Mazzoli, 2018). Escamilla Hurtado et al. (2005) reported the production of diacetyl with *Pediococcus pentosaceus* and *Lactobacillus acidophilus* increases on starchy substrates according to different parameters.

Moreover, the use of Kefir (granules containing lactic acid and acetic bacteria and fermenting yeasts) in SSF on food industry wastes showed significant production of ε-pinene (Table 1), with an estimated yield in biorefineries of 4 Kg per ton of treated substrate (Aggelopoulos et al., 2014).

Conclusions

Recent years have witnessed a rapid evolution of different methods of natural flavors and fragrance chemical production through biotechnological routes. The use of microbial cultures offers several advantages over traditional methodologies such as the possibility to label flavors as "natural" and thus making them more suitable for consumption, with market acceptability. Agri-food waste exploitation can be of great help, often offering excellent substrates for microbial growth and enhancing waste recovery and valorization at the same time. High operating costs are among the main issues to solve for the implementation of these production systems. The employment of LAB offers a possibility to add value to agri-food waste and produce natural flavors. Their use offers advantages compared to other microorganisms, such as GRAS status, good adaptability to different carbon sources and environmental stress, and disadvantages as they often need pretreatment to make sugar available and nutrient supplementations. FWs used as a substrate can overpass the last issue. Moreover, techniques of genetic modification in LAB are being rapidly developed thanks to the completion of genome sequencing and the wide availability of handling techniques. In light of the wide applicability of LAB fermentations, few studies have addressed the efficiency of this approach for the synthesis of aromatic compounds using agri-food waste as a substrate.

Undoubtedly, a careful assessment of production and downstream costs is mandatory to guarantee the economy of the design process. SSF technology has yet not been fully implemented at the industrial scale because of the lack of easily scalable re-actors, able to successfully overcome the problems with heterogeneity and sterility. Consequently, further targeted studies are needed to assess the most effective ways for the extraction and separation of flavor compounds at the industrial scale.

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Chapter 2

Exploit the potential of LAB on different by-products

Chapter 2.1

Production and recovery of volatile compounds from fermented fruit by-products with *Lacticaseibacillus rhamnosus*

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Abstract

In the last years, due to the increasing interest of consumers in natural products, market and industry choices were more directed towards the use of fragrances and flavors obtained from natural sources. Fermentation is a biological approach helpful to produce diverse aromatic compounds modifying precursors present in raw material, due to a wide array of extracellular enzymes which could be produced by microorganisms. Following this direction Lacticaseibacillus rhamnosus was employed as a starter for melon and orange by-products fermentation. Simple and vacuum distillation were applied for the recovery of the aromatic fraction and they were compared to identify the more promising compounds in terms of aromatic profile composition. Detection and characterization of aromatic compounds were achieved using Head SpaceSolid Phase MicroExtraction Gas Chromatography-Mass Spectrometry (HS-SPMEGC-MS) approach. Fermentation led to interesting changes, increasing typical citrus, orange, and melon aroma compounds. Overall, simple distillation allowed the greater recovery of aromatic compounds in terms of concentration for both substrates. The heating occurring during simple distillation has favoured aroma formation and compounds recovery but, at the same time, caused the degradation of thermolabile compounds and an incomplete collection of compounds produced during fermentation.

Introduction

Aromas and fragrances markets are in continuous development in various sectors from food, to feed, as to cosmetic and pharmaceutical industries. In 2019 the global sale of these compounds was approximately US$ 5.5 billion (Market analysis report, 2020). Aroma compounds can be obtained by several methods: chemical synthesis, extraction from vegetal matrices, and biotechnological processes. Disadvantages for chemical synthesis comprise pollution for using large volumes of organic solvents, low yield, lacking substrate selectivity which may cause the formation of undesirable racemic mixtures reducing process efficiency, and increasing cost (Akacha and Gargouri, 2015). In the last years, due to the increasing interest of consumers in natural products, market and industry choices were more and more directed towards the use of fragrances and flavors obtained from natural sources (Gupta et al., 2015).

Generally, the methods of extraction employed for aroma and pigments recovery from plant materials are represented by solvent extraction, hydro-distillation, steam distillation, and supercritical carbon dioxide extraction. Recently, enzymes have been used for the extraction of flavor and color from plants, as a pre-treatment of the raw material before subjecting it to hydro-distillation/solvent extraction (Sowbhagya and Chitra, 2010).

Other than using commercial enzymes, fermentation is a biological approach that is expected to produce diverse aroma compounds and modify precursors, due to a wide array of extracellular enzymes, especially hydrolases, that could be produced by microorganisms (McFeeters, 2004). Nowadays the industries are developing new biotechnological processes to obtain aroma compounds naturally. One of them is based on microbial bioconversion using solid-state fermentation (SSF).

SSF involves the cultivation of microorganisms on solid material which acts as physical support and a source of nutrients, in the absence of a free aqueous phase. This bioprocess offers several advantages such as low cost, simple equipment, and most important, it permits the use of agricultural and agro-industrial by-products as substrates (Martinez-Avila et al., 2014). The use of agro-industrial by-products as the culture medium for microorganisms represents a potentially significant reduction in the cost of bioprocesses (de

Olivera Felipe et al., 2017) and the transformation of the agri-food residues into products with commercial added value (dos Santos Barbosa et al., 2008).

Some studies have reported the use of SSF to produce aromatic compounds starting from agro-industrial by-products such as green coconut husk (dos Santos Barbosa et al., 2008), sugarcane bagasse (Guimarães et al., 2009), and coffee pulp (Orozco et al., 2008).

While much of the focus to date has centered on the substrates used, the exploitable microbial potential remains to be explored in depth. In particular, few studies have been carried out to produce flavor molecules using lactic acid bacteria (LAB), despite their aromatic potential is known during the fermentation of dairy and fruit products (Bancalari et al., 2020; Filannino et al., 2013; Levante et al., 2020; Ricci et al., 2018). Recently, the use of specific strains of *Lacticaseibacillus rhamnosus* in fruit fermentation has revealed a significant increase in the concentration of total volatile compounds and peculiar flavor signature (Ricci et al., 2018, 2019b). We therefore assessed the ability of this strain to grow on fruit by-products (melon and orange) and the recovery of the aromatic fraction. Two extraction techniques, simple and vacuum distillation, were compared to identify the more promising compounds in terms of aromatic profile composition. Detection and characterization of aromatic compounds were achieved using Head Space-Solid Phase Microextraction/Gas Chromatography-Mass Spectrometry (HS-SPME/GC-MS) approach.

Materials and methods

Substrates

Melon (*Cucumis melon*) by-products were collected from Azienda Agricola Ranieri, Colorno, Parma (Italy) and were composed of fruits discarded during harvest for inappropriate size, ripening and/or inadequate for shelling. Orange (*Citrus sinensis*) by-product (orange pomace) from Washington Navel variety were recovered after juice extraction. Each by-product was blended and kept at -20°C until their use.

Lactic acid fermentation processes

L. rhamnosus 1473 isolated from Parmigiano Reggiano cheese (collection of Food and Drug Department, University of Parma, Italy) and maintained at -80°C in De Man, Rogosa and Sharpe (MRS) broth (Oxoid, Basingstoke, UK) supplemented with 12.5% glycerol (v/v), was

used as starter for fermentation. Before fermentation, the strain was transferred twice in MRS broth (3% v/v) and incubated for 24 h at 37°C. Afterward, 200 μl of culture broth was inoculated in 6 mL of MRS broth and incubated at 37°C for 15 h to obtain a cell concentration of 9 Log CFU/mL. Bacterial culture was centrifuged (10,000 rpm, 10 min, 4 °C), washed twice in Ringer solution (VWR, UK), and suspended in sterile bidistilled water.

The concentration of endogenous microbiota in raw by-products was determined by plate count using PCA (VWR, Milan, Italy), and plates were incubated for 72 h at 30°C. Before fermentation, both substrates were sterilized (121°C for 20 min) and residual contamination was checked by plate count in PCA incubated at 30°C for 72 h. Sterile by-products were inoculated in triplicate in glass jars with the bacterial suspension to obtain a final concentration of 7 Log CFU/g, then the jars were closed with the cap and incubated at 37 °C for 72 h. The concentration of *L. rhamnosus* 1473 was checked just after inoculum (T0) and at the end of fermentation (T72) by plate count on MRS agar (Oxoid, Basingstoke, UK) (37°C for 48 h).

Recovery of aroma compounds by simple distillation

To recover aromatic fractions, fermented and unfermented samples were extracted by simple distillation (Lukin et al., 2018). Briefly, 200 g of melon by-products or 150 g of orange pomace were added with 50 g of sterile distilled water, placed in a 250 mL glass boiler on a hotplate and warmed at 100°C. The boiler was connected to a condenser, water was circulated for cooling vapors and the condensate was recovered in a flask. All the process was performed for 30 min. The samples containing aromatic components were kept at -80°C until analysis.

Recovery of aroma compounds using vacuum

Vacuum distillation was simulated by the use of a rotary evaporator (Strike 300, Steroglass, Italy). In particular, 75 g of fermented or unfermented orange pomace and 25 g of water were used. Aroma compounds dispersed in water, separated from the rest of the sample, were recovered at 40°C and 150 rpm until the complete evaporation of water. For melon by-products, the same procedure was followed starting from 100 g of fermented or

unfermented samples without adding water. All the recovered samples were stored at -80 °C until analysis.

Characterization of volatile profile with HS-SPME/GC-MS

The volatile fraction obtained through the use of rotavapor and simple distillation was characterized by the HS-SPME/GC-MS technique following the protocol reported by Ricci et al. (2018). For the analysis, 2 mL of samples were placed in 20 mL glass vials by adding 10 μL of a standard solution of toluene in water (100 μg / mL in 10 mL). The solid-phase microextraction of the headspace was carried out for 30 min at 40°C after an equilibration time of 15 min. For each analysis, a three-phase fiber for SPME (Supelco, Bellefonte, PA, USA) coated with 50/30 μm of Divenylbenzene-Carboxen-Polydimethylsiloxane (DVB / Carboxen / PDMS) was used.

GC-MS analysis was performed using a Thermo Scientific Trace 1300 gas chromatograph coupled to a Thermo Scientific ISQ single quadrupole mass spectrometer equipped with an electron impact source. The analysis was carried out for 45 min.

The acquisition of the signal was carried out in full scan mode (from 41 m / z to 500 m / z). The main volatile compounds were identified based on their mass spectrum compared with the mass spectra library (NIST). Moreover, to obtain a certain identification of the analytes found, linear retention indices or "Kovats indices" were calculated based on the retention times of a standard mixture of alkanes (C6 - C23).

Statistical Analysis

To verify differences in the production and/or release of aromatic compounds among the considered samples, data obtained from HS-SPME/GC-MS analyses were submitted to one-way ANOVA test applying Tukey's post hoc test ($p< 0.05$), using IBM SPSS Statistics 23.0 software (SPSS Inc., Chicago, IL). In addition, to investigate possible interactions between two variables, fermentation and recovery method, two-way ANOVA test was applied (Tukey's post hoc test with $p<0.05$).

Results and discussion

Fermentation of fruit by-products

To evaluate microbial contamination of the substrates, a total microbial count was performed before fermentation. Results indicated a similar concentration for the two matrices: 5.48±0.02 Log CFU/g in orange pomace and 5.52±0.11 Log CFU/g in melon by-product. A sterilization step allowed to eliminate the endogenous microflora and evaluate the growth ability of *L. rhamnosus* 1473. After 72 h of fermentation, it was recorded an increase in microbial cell number of 2.23 Log CFU/g and 2.31 Log CFU/g from the original inoculum in orange pomace and melon respectively. Despite the dairy origin of this strain, the ability to grow in plant substrates is in agreement with previous studies (Ricci et al., 2020, 2019c, 2019a).

Effect of fermentation and recovery techniques on aroma concentration

Orange pomace

Characterization of the volatile profile of unfermented and fermented orange pomace obtained after simple and vacuum distillation was carried out using the HS-SPME/GC-MS technique. All the volatile molecules detected belong to the following chemical classes: ketones, alcohols, aldehydes, esters, terpenes and derivatives, and norisoprenoids (Appendix A. Supplementary data Table A1 and Table A2).

In particular, 82 compounds were identified and semi-quantified in samples obtained by simple distillation, while 97 compounds were characterized in samples analyzed after simulated vacuum distillation. The characteristic odor type for each molecule was also defined (Table 1).

Table 1. Concentration of different compounds (μg/mL) in distillates of fermented and unfermented orange pomace obtained with vacuum distillation using rotavapor and simple distillation. * indicates significance at $p<0.05$. n.d not detected

	Vacuum distillation		**Simple distillation**	
Odor type	Unfermented	Fermented	Unfermented	Fermented
Solvent				
Acetone	0.02±0.02	0.34±0.31	0.40±0.16	0.08±0.02*
Alcoholic				
Ethanol	n.d	0.29±0.25	n.d	n.d
Aldehydic				
3-Methyl-butanal	n.d	n.d	0.28±0.16	0.14±0.06
Octanal	n.d	0.01±0.00*	0.18±0.00	0.04±0.01*
Decanal	0.01±0.00	0.04±0.01*	0.41±0.02	0.13±0.04*

Dodecanal	n.d	n.d	0.22±0.01	1.02±1.01
Buttery				
Acetoin	0.11±0.01	0.45±0.06*	n.d	n.d
Diacetyl	n.d	n.d	0.03±0.00	0.09±0.01*
Fermented				
3-Methyl-1-Butanol	n.d	0.22±0.02*	n.d	n.d
Herbal				
3-Buten-2-ol, 2-methyl-	0.02±0.01	0.08±0.02*	n.d	n.d
Herbal ketone	0.01±0.00	0.06±0.02*	n.d	n.d
Safranal	0.03±0.00	0.04±0.03	0.66±0.02	0.52±0.03*
1R-α-Pinene	n.d	n.d	0.01±0.00	n.d*
2-Methyl-3-buten-2-ol	n.d	n.d	0.06±0.04	0.10±0.01
β-Pinene	n.d	n.d	0.03±0.03	n.d
1-Hexanol	n.d	0.03±0.03	0.06±0.04	1.12±0.13*
Perillaldehyde	n.d	n.d	0.30±0.04	0.25±0.02
Isopiperitenone	0.01±0.00	0.06±0.02*	n.d	n.d
Green				
Hexanal	0.01±0.00	n.d	0.036±0.00	0.02±0.01*
Heptanal	0.01±0.00	0.02±0.02	0.014±0.01	0.02±0.02
p-Mentha-1,8-dien-7-ol	0.02±0.00	0.12±0.03*	n.d	n.d
1-Heptanol	n.d	n.d	0.036±0.00	0.09±0.02*
Perillic alcohol	n.d	n.d	0.265±0.03	0.22±0.14
Citrus				
Limonene	0.02±0.01	0.10±0.06	4.77±1.25	2.33±1.36
2-Ethyl-1-Hexanol	n.d	0.01±0.00	n.d	n.d
Sulcatone	n.d	n.d	0.04±0.00	0.07±0.01*
Citral	0.14±0.01	0.94±0.13*	1.06±0.21	0.59±0.20
Valencene	0.03±0.01	0.20±0.03*	2.03±0.11	2.15±0.48
β-Sinensal	0.06±0.00	0.26±0.04*	0.82±0.03	0.61±0.27
Terpenic				
γ-Terpinene	0.02±0.00	0.44±0.08*	0.14±0.02	n.d*
α -Terpineol	0.34±0.00	1.51±0.21*	n.d	n.d
1,3,8-p-Menthatriene	n.d	n.d	0.16±0.15	0.20±0.08
Fruity				
Prenol	n.d	n.d	0.25±0.03	0.21±0.03
Benzaldehyde	0.01±0.00	0.01±0.01	0.14±0.03	0.06±0.04
Hexanoic acid, 3-hydroxy-, ethyl ester	0.02±0.00	0.25±0.20	n.d	n.d
p-Menth-1-en-9-ol	n.d	n.d	0.90±0.10	0.95±0.11
β-Cyclocitral	n.d	n.d	1.38±0.23	1.23±0.29
Ethyl 3-hydroxyhexanoate	n.d	n.d	0.19±0.02	0.30±0.09
2-Cyclopentyl cyclopentanone	n.d	n.d	0.39±0.18	0.16±0.14
Floral				
(cis)-Rose oxide	n.d	n.d	n.d	0.02±0.00*
β-Linalool	0.08±0.01	1.12±0.27*	1.63±0.29	2.31±0.37
Lilac aldehyde	0.00±0.00	0.01±0.00*	n.d	n.d
1-Nonanol	0.01±0.00	0.14±0.04*	0.45±0.07	0.68±0.02*
Citronellyl formate	n.d	n.d	0.31±0.25	1.63±0.96
Citronellol	0.03±0.00	0.34±0.09*	n.d	n.d

p-Methylacetophenone	n.d	n.d	0.11±0.00	0.17±0.04
Nerol	0.02±0.00	0.26±0.08*	n.d	n.d
Geraniol	0.04±0.00	0.29±0.08*	n.d	n.d
cis-Geraniol	n.d	n.d	0.18±0.08	0.42±0.10*
trans-geraniol	n.d	n.d	0.30±0.07	2.44±0.28*
Geranyl acetone	n.d	n.d	0.28±0.04	0.37±0.00*
Benzyl alcohol	0.04±0.01	0.26±0.02*	n.d	n.d
trans-Nerolidol	0.01±0.00	0.04±0.01*	n.d	n.d
Nerolidol	n.d	n.d	0.20±0.00	0.09±0.06*
Ethanone. 1-(2-hydroxy-5-methylphenyl)-	n.d	n.d	1.44±0.08	0.18±0.01*
Waxy/Fatty				
Methyl Octanoate	n.d	n.d	n.d	0.02±0.02
(E)-2-Nonenal	n.d	n.d	0.06±0.01	0.03±0.03
1-Octanol	0.07±0.01	1.48±0.49*	1.75±0.52	2.05±1.49
1-Decanol	0.04±0.01	0.32±0.09*	0.91±0.53	1.99±0.65
1- Dodecanol	0.01±0.00	0.03±0.01*	n.d	n.d
Isopropyl myristate	0.01±0.00	0.07±0.01*	n.d	n.d
γ-Eudesmol	0.01±0.00	0.02±0.01	n.d	n.d
Hexadecanoic acid, methyl ester	0.01±0.00	0.04±0.01*	n.d	n.d
Woody				
2-Menthoxy-4-vinyphenol	0.21±0.03	0.26±0.04	n.d	n.d
β-Isophorone	n.d	n.d	0.06±0.00	0.01±0.00*
α-Copaene	n.d	n.d	0.08±0.00	0.02±0.01*
Caryophyllene oxide	n.d	n.d	0.08±0.00	0.05±0.01*
Spicy				
(-)-4-Terpineol	n.d	n.d	1.37±0.29	1.58±0.25
4-Terpineol	0.14±0.01	1.16±0.26*	n.d	n.d
(+)-trans-Carveol	n.d	n.d	0.15±0.15	0.41±0.05
(+)-cis-Carveol	n.d	n.d	3.38±0.39	1.44±1.27
(-)-cis-Carveol	0.78±0.03	2.79±0.35*	1.46±o.28	0.33±0.08*
(-)-trans-Carveol	n.d	n.d	0.42±0.08	0.86±0.36
Carveol	0.26±0.00	1.05±0.20*	n.d	n.d
Eugenol	0.02±0.00	0.16±0.05*	n.d	n.d
Minty				
p-Menth-8-en-2-one	n.d	0.02±0.00*	n.d	n.d
Menthol	n.d	0.01±0.01	n.d	n.d
Dihydrocarvone	n.d	n.d	0.09±0.02	0.89±0.32*
cis-p-Mentha-2,8-dien-1-ol	0.02±0.00	0.18±0.16	0.21±0.01	0.11±0.02*
Eucarvone	n.d	n.d	1.32±0.26	0.92±0.07
Carvone	0.04±0.01	0.13±0.03*	0.57±0.05	0.12±0.09*
Piperitenone	0.01±0.00	0.04±0.01*	n.d	n.d
Bready/caramellic				
Furural	0.76±0.03	0.54±0.05*	2.77±0.01	0.58±0.38*
5-Methylfurfural	0.08±0.00	0.05±0.05	0.42±0.01	0.23±0.08*
Phenolic/balsamic				
2-p-Tolylpropene	0.01±0.00	0.04±0.00*	0.27±0.26	0.51±0.50
2-Acetylfuran	n.d	n.d	0.13±0.00	0.05±0.03*
Methyl benzoate	0.01±0.00	0.07±0.01*	n.d	n.d

Butylated hydroxytoluene	0.05±0.02	0.03±0.01	n.d	n.d
Camphoreous				
1,5,7-Octatrien-3-ol,2,6-dimethyl	0.01±0.00	0.06±0.02*	n.d	n.d
Earthy				
Spathulenol	0.01±0.00	0.03±0.01*	n.d	n.d
Sulfurous				
Dimethyl sulfide	n.d	n.d	0.01±0.00	0.02±0.00*

The concentrations calculated for fermented and unfermented samples, recovered by both the extraction techniques (Table 1), were submitted to a first statistical analysis (two-way ANOVA test) to verify the interaction between two factors, fermentation process and recovery method, and to identify which variable influenced the composition of an aromatic fraction more prominently. Results are shown in Table 2.

Table 2. Concentration of different odor types in orange pomace (μg/mL). Data are expressed as mean ± standard deviation. C is the unfermented sample; D is simple distillates; F is the fermented sample and R means distillation by rotavapor. C/F indicates differences between unfermented (C) and fermented (F) samples; D/R indicates differences between simple (D) and vacuum (R) distillation; CF*DR explains the interaction between the factors. n.d not detected.

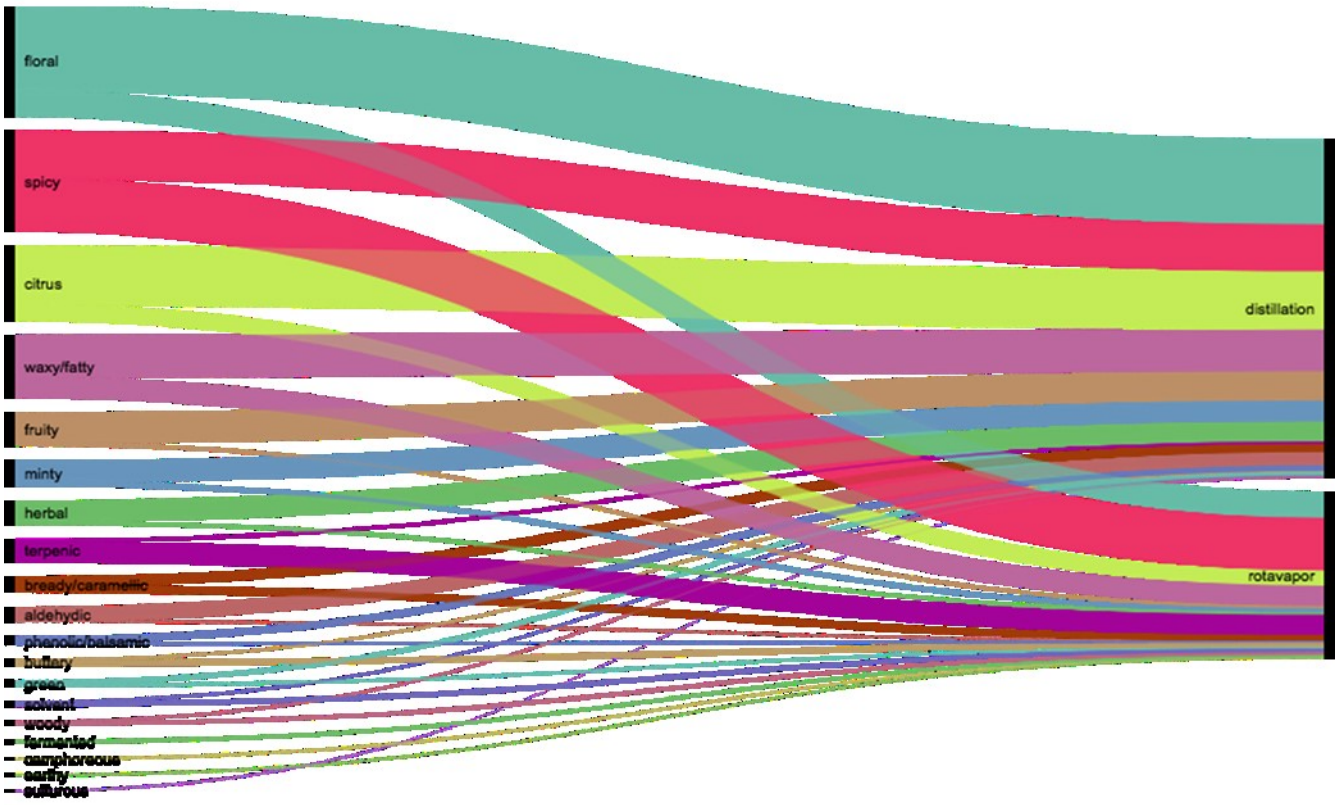
	Concentration (μg/mL)				**two-way ANOVA (p<0.05)**		
	C/D	**F/D**	**C/R**	**F/R**	**C/F**	**D/R**	**CF*RD**
Solvent	0.40±0.16	0.07±0.02	0.02±0.02	0.33±0.30	0.94	0.55	0.01
Sulfurous	0.01±0.00	0.02±0.00	n.d	n.d	0.00	0.00	0.00
Aldehydic	1.11±0.17	1.31±0.98	0.00±0.00	0.04±0.01	0.67	0.00	0.77
Buttery	0.03±0.00	0.09±0.01	0.11±0.00	0.44±0.05	0.00	0.00	0.00
Herbal	1.14±0.13	1.98±0.09	0.07±0.01	0.27±0.05	0.00	0.00	0.00
Green	0.35±0.05	0.35±0.15	0.03±0.00	0.14±0.04	0.28	0.00	0.29
Citrus	8.73±0.94	5.75±1.88	0.24±0.00	1.51±0.26	0.20	0.00	0.00
Terpenic	0.30±0.13	0.20±0.07	0.36±0.00	1.95±0.29	0.00	0.00	0.00
Fruity	3.28±0.03	2.91±0.44	0.02±0.00	0.26±0.18	0.64	0.00	0.05
Floral	4.93±0.75	8.29±1.78	0.23±0.02	2.45±0.59	0.00	0.00	0.35
Waxy/fatty	2.73±1.08	4.09±0.79	0.15±0.01	1.96±0.61	0.00	0.00	0.61
Woody	0.23±0.00	0.08±0.00	0.20±0.02	0.26±0.04	0.01	0.00	0.00
Spicy	6.80±0.90	4.61±1.19	1.19±0.01	5.16±0.85	0.11	0.00	0.00
Minty	2.20±0.36	2.04±0.28	0.07±0.00	0.37±0.19	0.66	0.00	0.15
Bready/caramellic	3.20±0.03	0.81±0.29	0.84±0.036	0.59±0.00	0.00	0.00	0.00
Phenolic/balsamic	0.41±0.26	0.55±0.53	0.07±0.01	0.13±0.01	0.56	0.05	0.82
Alcoholic	n.d	n.d	0.00±0.00	0.29±0.24	0.07	0.07	0.07

Ethereal	n.d	n.d	0.00±0.00	0.00±0.00	0.25	0.03	0.25
Fermented	n.d	n.d	0.00±0.00	0.22±0.02	0.00	0.00	0.00
Camphoreous	n.d	n.d	0.01±0.00	0.05±0.01	0.00	0.00	0.00
Earthy	n.d	n.d	0.00±0.00	0.03±0.01	0.01	0.01	0.01
Total	35.92± 2.40	33.22±6.59	3.70±0.10	16.56±2.67	0.54	0.00	0.351

Data highlighted that the concentration of total volatiles observed in the distillates of fermented and unfermented orange pomace was different depending on the recovery method. In general, it has been shown that the aroma was influenced by both fermentation and recovery methods, and in most cases, it was observed an interaction of the factors considered ($p<0.05$). This means that for some odor types, both factors influence the concentration detected. With simple distillation, the quantity of recovered compounds did not differ between the fermented (33.22 ± 6.59 μg/mL) and unfermented sample (35.92 ± 2.40 μg/mL), while the recovery with the vacuum showed significant differences ($p<0.05$) between unfermented and fermented by-product, where the total of compounds is equal to 3.70 ± 0.10 μg/mL and to 16.56 ± 2.67 μg/mL respectively.

Differences between the classes of compounds and odor type identified for the two distillates obtained from orange pomace were reported in Table 1 and represented in Figure 1.

Figure 1. Alluvial diagram of fermented orange pomace odor type obtained with simple distillation and using rotavapor



A greater recovery of aromatic compounds, with a wider different odor type, was found after simple distillation in comparison to vacuum one. This could be related to the techniques applied, and in particular to the temperature at which the recovery takes place. Since simple distillation was performed around 100°C, heating has increased the formation and recovery of aroma compounds, that were present to a less extent after vacuum because it occurred at low temperatures (Sarrazin et al., 2000). Although simple distillation is the procedure that leads to a higher extraction yield, no significant differences were noted between the unfermented and fermented samples, which instead are evident for those subjected to vacuum distillation. Therefore, based on these results, it is possible to state that the use of high temperatures seems to minimize the contribution given to the aroma by the fermentation process. Moreover, differences could be based on the preference of some volatile compounds for one process according to their hydrophobicity and volatility. The individual aroma components differ according to their molecular structure, which in turn defines the solubility, the boiling point, and the volatility of each type of compound (Bagger-

Jørgensen et al., 2004). In particular, the sample obtained from simple distillation is characterized by floral, citrus, waxy, and fruity notes, while the other is represented by spicy and terpenic odor types (Table 2).

Floral notes, that are predominant in fermented samples subjected to simple distillation (Figure 1), seem to be influenced by fermentation and also by the recovery method even if the interaction among these factors didn't significantly affect these notes. Various studies reported the role of LAB that improved these floral notes in different fermented products such as fruit juice (Di Cagno et al., 2017; Ricci et al., 2018), wine (Du Plessis et al., 2019), cocoa beans (Viesser et al., 2021), coffee (de Melo Pereira et al., 2020), and wheat bran (Spaggiari et al., 2020).

In the present study floral notes derive from (cis)-Rose oxide, β-linalool, 1-nonanol, citronellyl formate, trans and cis-geraniol, and nerolidol. Citronellyl formate is a terpene found in citrus fruits, in essential oils of geranium, mandarin, and also in honey. The increase of this compound has already been observed after fermentation with *L. plantarum* in the bog blueberry (*Vaccinium uliginosum*) (M. Wei et al., 2018). The increase of trans-geraniol, acyclic monoterpenic alcohol was significant after fermentation. It is found in different varieties of orange (Selli and Kelebek, 2011; X. Wei et al., 2018) and is a common constituent of many essential oils like rose oil. Another compound that showed an increase after fermentation is linalool. This monoterpene, characterized by floral attributes and low threshold values, is released by the hydrolysis of linalyl β-D-glycoside (Maicas and Mateo, 2005). Several studies, in accordance with the data reported in this study, have highlighted its increase after lactic acid fermentation in different matrices (Ricci et al., 2018; Zhou and Mcfeeters, 1998). The increase of specific terpene compounds may be due to their *ex novo* synthesis, as secondary metabolites, by some bacteria strains (Di Cagno et al., 2009) or to the hydrolytic action of bacteria on terpenes glycosylates with the release of the aglyconic fraction (Ricci et al., 2018).

The recovery of molecules characterized by citrus notes was influenced by both fermentation and the extraction method. Molecules responsible for citrus notes were limonene, citral, and valencene but no statistical differences were observed in concentration

after fermentation. In particular, all these compounds presented higher concentrations in samples obtained by simple distillation in respect to those recovered by vacuum. Citral and valencence amounts resulted were influenced by fermentation process (Table 1), and this resulted evident in samples derived from vacuum distillation. So, for these notes, it can be underlined as simple distillation affect the contribution given by fermentation.

Fruity notes are represented by benzaldehyde, p-Menth-1-en-9-ol, β-Cyclocitral, Ethyl 3-hydroxyhexanoate, and 2-cyclopentyl-cyclopentanone. In this case, differences were observed only for the type of extraction and were not influenced by fermentation (Table 1 and 2). The greatest recovery occurred with simple distillation.

The herbaceous compounds, especially 1-hexanol, were positively affected by fermentation and recovered mainly by simple distillation. Also these types of notes were previously reported after the growth of LAB in products such as fermented elderberry juice (Ricci et al., 2018) and cocoa beans (Viesser et al., 2021). Moreover, fermentation increased the buttery notes, which were recovered mainly with vacuum distillation (Table 2). Buttery notes, typical of LAB fermentation, were previously found in fermented beverages like wine (Zhou and Mcfeeters, 1998), fermented elderberry juice (Ricci et al., 2018) and dairy products (Bancalari et al., 2020).

Bready and caramelly notes, prevalent due to furfural presence, stood out in unfermented orange pomace after distillation, while were softened after fermentation. This molecule originates after heating treatment, during the Maillard reaction and caramelization (Kroh, 1994).

Fermented orange pomace distillates obtained with vacuum had a high concentration of compounds with terpenic and spicy odor type. Molecules with terpenic notes are γ-terpinene and α-terpineol, which showed a significant increase after fermentation. As reported by a previous study, α-terpineol may be generated from the degradation of D-limonene or resulting from the hydrolysis of volatile glycosidic precursors in oranges during fermentation (Gang et al., 2009).

Spicy notes are detected for the presence of 4-terpineol, carveol, cis-carveol, and eugenol. The concentration of these molecules increased after fermentation ($p<0.05$). In particular, the

methods of recovery and fermentation increased the concentration of these compounds in the vacuum distillates. 4-terpineol naturally found in fruits as apple, apricot, orange, and lemon is a food additive permitted for direct addition, and its increase after fermentation has already been reported (Fan et al., 2009). The increase in eugenol has been previously reported in the literature in elderberry juice after lactic acid fermentation with the same strain of *L. rhamnosus* employed in this study (Ricci et al., 2018). This compound, present in clove essential oil, is known for its antioxidant properties and exerts an antimicrobial effect against different pathogens (Devi et al., 2010).

Melon by-products

From the characterization of the volatile profile of unfermented and fermented melon by-products carried out using the HS-SPME/GC-MS technique, 99 compounds were identified after simple distillation and 79 after vacuum recovery. These molecules pertain to ketones, alcohols, aldehydes, esters, terpenes and derivatives, and norisoprenoid categories (Appendix A. Supplementary data Table A3 and Table A4).

Table 3 shows the total concentrations of the different compounds. As expected, differences can be observed between unfermented and fermented samples. Figure 2 shows the different odor type of these molecules and it is highlighted how the distillate obtained by simple distillation permitted a more abundant recovery in aromatic compounds.

Table 3. Concentration of different compounds (μg/mL) in distillates of fermented and unfermented melon by-products obtained with vacuum distillation using rotavapor and simple distillation. * indicates significance at $p<0.05$. n.d not detected

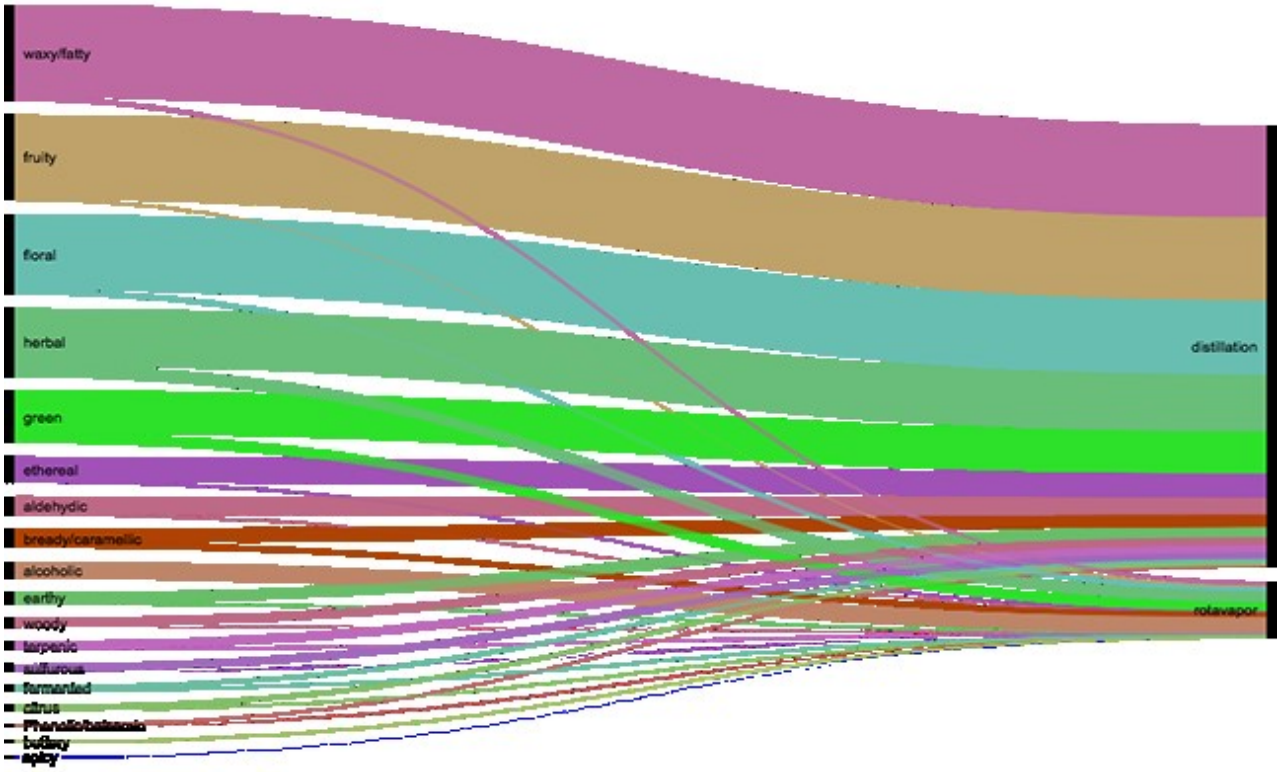
	Vacuum distillation		**Simple distillation**	
Odor type	Unfermented	Fermented	Unfermented	Fermented
Ethereal				
Acetaldehyde	0.16±0.15	0.02±0.01	1.32±0.08	0.47±0.06*
Ethyl Acetate	0.47±0.43	0.06±0.01	0.51±0.01	7.45±1.72*
1-Propanol, 2-methyl	0.08±0.04	0.31±0.13	0.88±0.17	1.90±0.13*
Alcoholic				
Ethanol	1.29±0.12	6.53±0.40*	n.d	n.d
Sulfurous				
Methyl 2-(methylthio)acetate	0.02±0.01	0.03±0.03	n.d	n.d
Disulfide dimethyl	n.d	n.d	0.43±0.04	1.25±0.54
Dimethyl trisulfide	n.d	n.d	0.83±0.17	1.35±1.09

Aldehydic				
Butanal, 3-methyl	n.d	n.d	0.77±0.05	0.89±0.85
Octanal	n.d	n.d	0.21±0.02	0.08±0.06*
Nonanal	0.25±0.15	0.12±0.04	1.98±1.72	3.55±3.53
Decanal	0.07±0.00	0.04±0.02*	0.22±0.03	0.53±0.04*
Undecanal	n.d	n.d	0.07±0.00	2.01±0.68*
Buttery				
Acetoin	1.19±0.14	0.50±0.18*	n.d	n.d
Herbal				
Eucalyptol	n.d	n.d	0.74±0.34	1.96±0.82
1-Hexanol	0.40±0.25	4.34±1.89*	10.20±1.95	21.17±1.63*
Green				
Hexanal	0.4±0.01	0.07±0.04	0.30±0.11	0.52±0.10
Heptanal	n.d	n.d	0.02±0.01	0.06±0.01*
2-Butenal, 2-methyl	0.03±0.03	0.02±0.01	n.d	n.d
2-Hexenal, E	n.d	n.d	1.69±1.24	8.58±6.07
3-Hexen-1-ol,(Z)	0.84±0.37	2.80±1.38	3.75±2.98	3.51±3.16
Hexanoic acid, hexyl ester	n.d	n.d	0.02±0.01	0.39±0.06*
1-Heptanol	n.d	0.19±0.08*	0.77±0.13	2.53±0.19*
Propanal,3-(methythio)	0.07±0.00	0.05±0.03	n.d	n.d
2,6-Nonadienal,E,Z	0.07±0.01	0.08±0.05	0.13±0.08	0.89±0.15*
5-Octen-1-ol	0.06±0.02	0.20±0.08*	n.d	n.d
Benzeneacetic acid, 2-hexenyl ester	0.19±0.19	0.09±0.04	n.d	n.d
3,6-Nonadien-1-ol	0.07±0.02	0.09±0.03	n.d	n.d
2-Phenyl-2-butenal	0.08±0.02	0.05±0.03	n.d	n.d
Ethyl trans-4-decenoate	n.d	n.d	0.04±0.03	0.60±0.06*
Citrus				
D-Limonene	0.01±0.00	0.14±0.05*	n.d	n.d
5-Hepten-2-one,6-methyl	n.d	n.d	0.29±0.28	0.65±0.07
2-Ethyl-1-hexanol	n.d	0.06±0.03*	0.15±0.01	0.80±0.17*
Terpenic				
Cyclohexanone,2,2,6-trimethyl	n.d	n.d	0.24±0.03	0.93±0.30*
α-Terpineol	0.18±0.04	0.25±0.07	0.95±0.26	1.99±0.16*
Fruity				
Butanoic acid, ethyl ester	0.07±0.05	0.02±0.01	0.70±0.09	8.76±1.28*
Butanoic acid,2-methyl-, ethyl ester	0.03±0.02	0.03±0.01	0.45±0.26	2.31±2.23
Butanoic acid, 2-methyl-ethyl ester	n.d	n.d	0.99±0.02	9.80±2.15*
Butanoic acid, methyl ester	n.d	n.d	0.79±0.73	2.98±2.89
Butanoic acid, 3-hydroxy-, ethyl ester	0.02±0.00	0.05±0.00*	n.d	n.d
2-Pentenal, 2-methyl	n.d	n.d	0.26±0.04	0.19±0.01
Butanoic acid, 2-methyl-2-methylpropyl ester	n.d	n.d	0.16±0.02	0.58±0.35
Hexanoic acid, ethyl ester	n.d	n.d	0.01±0.00	0.05±0.04
Acetic acid, hexyl ester	n.d	n.d	0.28±0.02	1.68±1.25
Butanoic acid, 2-methyl-2-methylbutyl ester	n.d	n.d	0.15±0.15	1.17±0.76

3-Hexenoic acid, ethyl ester	n.d	n.d	0.15±0.07	1.04±0.72
Hexanoic acid, butyl ester	n.d	n.d	0.03±0.02	0.07±0.06
Isopentyl hexanoate	n.d	n.d	0.14±0.11	1.35±1.33
2.4-Hexadienoic acid, ethyl ester	n.d	n.d	0.03±0.02	1.07±0.04*
Benzaldehyde	1.26±0.39	0.39±0.15*	7.76±2.03	2.88±0.01*
6-Nonenol	0.08±0.02	0.09±0.03	n.d	n.d
Floral				
Acetic acid, octyl ester	n.d	n.d	0.29±0.25	0.93±0.33
Linalool	n.d	n.d	0.48±0.15	4.43±1.67*
β-Linalool	0.02±0.02	0.09±0.00*	n.d	n.d
1-Nonanol	0.06±0.01	0.24±0.87*	0.86±0.86	5.32±3.34
Geraniol	0.02±0.00	0.11±0.04*	n.d	n.d
5,9-Undecadien-1-one, 6,10-dimethyl	0.10±0.00	0.11±0.02	1.83±0.04	3.48±0.92*
β-Damascenone	0.03±0.00	0.12±0.03*	1.21±0.11	8.01±1.28*
Benzyl Alcohol	1.01±0.12	0.83±0.13	3.02±0.14	4.13±0.07*
Phenylethyl Alcohol	0.16±0.00	0.20±0.03	0.77±0.06	1.72±0.18*
α-Ionone	n.d	n.d	1.45±0.06	2.35±0.31*
trans-β-Ionone	0.05±0.01	0.07±0.01	n.d	n.d
Waxy/Fatty				
(E)-2-Octenal	n.d	n.d	0.01±0.00	1.45±0.03
Octanoic acid, ethyl ester	n.d	n.d	0.15±0.11	4.90±4.72
(E)-2-Nonenal	0.03±0.03	0.05±0.03	0.36±0.10	1.13±0.61
1-Octanol	0.15±0.03	1.08±0.36*	7.05±1.51	21.55±0.20*
Decanoic acid, ethyl ester	0.16±0.02	0.15±0.04	0.05±0.01	1.42±1.27
(E)-2-Decenal	n.d	n.d	0.36±0.16	5.38±2.60*
Dodecanoic acid, ethyl ester	n.d	n.d	0.63±0.02	1.18±0.24*
1-Decanol	n.d	0.01±0.00*	n.d	n.d
5-Decen-1-ol	0.02±0.01	0.04±0.01*	n.d	n.d
Ethyl laurate	0.12±0.01	0.04±0.01*	n.d	n.d
1-Dodecanol	0.06±0.05	0.04±0.01	n.d	n.d
Isopropyl myristate	0.10±0.05	0.04±0.01	n.d	n.d
Hexadecanoic acid, ethyl ester	0.10±0.01	0.06±0.03	n.d	n.d
Woody				
1-Cyclohexene-1-acetaldehyde, 2,6,6-trimethyl	n.d	n.d	1.32±0.13	2.50±1.46
Cadina-3,9-diene	n.d	n.d	0.08±0.07	0.06±0.06
α-Cadinene	n.d	n.d	0.04±0.02	0.28±0.10*
β-Cadinene	0.07±0.00	0.05±0.02	n.d	n.d
2-Methoxy-4-vinylphenol	0.14±0.00	0.05±0.01*	n.d	n.d
2-Butanone, 4-(2,6,6-trimethyl-1-cyclohexen-1-yl)	n.d	n.d	1.13±0.29	0.26±0.06*
Spicy				
Eugenol	0.02±0.00	0.01±00*	n.d	n.d
Roasted/bready				

1-Butanol, 2-methyl	0.38±0.24	1.84±0.87*	2.67±2.14	5.15±4.47
Furfural	0.21±0.04	0.01±0.00*	n.d	n.d
Ethanone, 1-(2-pyridinyl)	0.09±0.01	0.04±0.00*	n.d	n.d
Balsamic				
3-Phenylpropanol	0.09±0.01	0.07±0.02	0.50±0.08	0.52±0.15
Fermented				
1-Butanol	0.19±0.10	0.39±0.19	0.05±0.02	0.38±0.04*
1-Pentanol	0.04±0.02	0.23±0.11*	0.16±0.15	1.05±0.32*
Earthy				
1-octen-3-ol	0.06±0.06	0.20±0.07	0.82±0.14	4.13±0.18*

Figure 2. Alluvial diagram of fermented melon by-product odor type obtained with simple distillation and using rotavapor



As observed for orange pomace, in most cases, the aroma was influenced by fermentation, recovery method and often also by the interaction of both factors considered ($p<0.05$) that affected the concentration observed. In simple distillation, as after the recovery with the rotavapor vacuum, fermented samples produced the highest concentration of recovery compounds. However, simple distillation was the technique that allowed to obtain the highest volatile concentration (Table 4).

Unlike the orange pomace, where the two distillates showed different odors depending on the type of technique used for the recovery, the two melon-based distillates differed only in the quantity of the same notes (Table 4). Both were characterized by fatty, fruity, floral, green, ethereal, aldehydic, and bready notes.

Table 4. Concentration of different odor type in melon by-product (μg/mL). Data are expressed as mean ± standard deviation. C is the unfermented sample; D simple distillates; F is the fermented sample and R means distillation by rotavapor. C/F indicates differences between unfermented (C) and fermented (F) samples; D/R indicates differences between simple (D) and vacuum (R) distillation; CF*DR explains the interaction between the factors. n.d not detected

	Concentration (μg/mL)				two-way ANOVA (p<0.05)		
	C/D	**F/D**	**C/R**	**F/R**	**C/F**	**D/R**	**CF*RD**
Sulfurous	1,25±0,21	2,59±1,63	0,02±0,01	0,03±0,02	1,00	0,00	0,20
Aldehydic	3,24±1,65	7,06±1,89	0,31±0,15	0,16±0,05	0,19	0,00	0,02
Buttery	n.d	n.d	1,19±0,14	0,49±0,18	0,03	0,00	0,00
Herbal	10,94±2,29	23,13±0,81	0,40±0,25	4,34±1,89	0,00	0,00	0,00
Green	6,70±1,68	17,09±2,32	1,46±0,65	3,63±1,76	0,00	0,00	0,00
Citrus	0,44±0,27	1,45±0,24	0,01±0,00	0,20±0,07	0,00	0,00	0,00
Terpenic	1,19±0,23	2,92±0,13	0,17±0,04	0,25±0,07	0,00	0,00	0,00
Fruity	11,89±3,27	33,94±7,91	1,46±0,48	0,56±0,17	0,00	0,00	0,00
Floral	9,90±0,91	30,36±7,43	1,45±0,07	1,77±0,34	0,00	0,00	0,00
Waxy/fatty	8,60±1,68	37,01±1,86	0,74±0,02	1,50±0,48	0,00	0,00	0,00
Woody	2,56±0,47	3,09±1,67	0,20±0,00	0,09±0,02	0,00	0,00	0,54
Spicy	n.d	n.d	0,02±0,00	0,01±0,00	0,68	0,00	0,00
Bready/caramellic	2,67±2,14	5,14±4,46	0,66±0,28	1,88±0,86	0,00	0,10	0,67
Phenolic/balsamic	0,50±0,08	0,51±0,14	0,09±0,01	0,06±0,01	0,24	0,00	0,67
Alcoholic	n.d	n.d	1,29±0,11	6,52±0,40	0,95	0,00	0,00
Ethereal	2,70±0,25	9,82±1,52	0,71±0,61	0,38±0,12	0,00	0,00	0,00
Fermented	0,20±0,17	1,43±0,36	0,22±0,11	0,61±0,30	0,00	0,03	0,02
Earthy	0,82±0,14	4,12±0,18	0,06±0,05	0,19±0,07	0,00	0,00	0,00
Total	63,67±11,75	179,72±21,46	10,53±2,22	22,74±6,88	0,00	0,00	0,00

It was observed that the molecules responsible for a peculiar odor type were different in function of the recovery method (Table 3). This confirms what has been said previously, that the type of distillate obtained with one or the other technique was influenced by the nature of the compounds naturally present in the substrate to be treated (Bagger-Jørgensen et al.,

2004). Depending on the type of molecule or the odor type to be extracted, a specific technique will be used. Moreover, several interactions between the fermentation process and the type of recovery were observed for different odor types (Table 4).

Overall, the predominant aromatic notes observed in melon by-products were waxy and fatty, fruity, floral, herbal, green, ethereal and aldehydic, and resulted higher in fermented samples (Table 3). As for orange pomace, the greatest recovery of aromatic compounds for melon by-products was observed in fermented and simple distilled samples.

Observing the molecules that have floral notes, it has been shown that the concentration of β-damascenone increased after fermentation with LAB as already reported in fermented fruit juice (Ricci et al., 2018) and fermented fruit extract (Schindler et al., 2012). The floral notes of β-damascenone increased after the recovery with simple distillation. Once again the type of recovery and fermentation affected the concentration. Fruity notes are represented especially by butanoic acid-ethyl ester and butanoic acid, 2-methyl-, ethyl ester. Both are used as flavoring agents and showed a significant increase after fermentation. The ethyl ester of butanoic acid is characterized by a fruity aroma with aromatic notes referable to those of pineapple and strawberry. This compound, resulting from the esterification of ethanol and butanoic acid, can be produced by many species of LAB of dairy origin (Liu et al., 1998). Molecules responsible for waxy and fatty notes, 1-Octanol and (E)-2-Decenal, showed a significant increase after fermentation. Octanol, a primary alcohol, finds its use in cosmetics, perfumery, as a flavoring agent and it is produced by different species of bacteria (Tait et al., 2014). (E)-2-decenal is an aldehyde naturally present in a wide range of fruits such as melon (Shalit et al., 2001), vegetables and spices, it plays a role as a flavoring and antibacterial agent. Its increase during fermentation with *L. rhamnosus* has already been reported in literature (Sgarbi et al., 2013) although in dairy matrices.

Among the molecules responsible for green notes, 2-hexenal, characteristic of melon, is an aromatic compound found in various types of fruit and vegetables (Schieberle et al., 1990). This compound, deriving from linoleic acid, has been studied for its antimicrobial properties both in vitro and in various products such as tea-based drinks, sliced apples in a modified atmosphere, and grapes, giving multiple advantages on shelf-life, color, and aroma (Gardini

et al., 2001), but its increase after lactic acid fermentation had not yet been reported in the literature.

Another compound known in literature to be characteristic of cucurbits, particularly concentrated in cucumber and also present in melon (Shalit et al., 2001), is 2,6-nonadienal (Schieberle et al., 1990), whose increase was also observed during the production and aging of wine (Matsui, 2006).

Ethyl acetate and acetic acid are the compounds which confer ethereal notes. They can be found in alcoholic beverages, fruit juices, beer, and wine and are often used in artificial fruit essences. Following lactic acid fermentation, these compounds showed an increase already observed in other studies on different matrices and also using LAB species other than L. rhamnosus (Ricci et al., 2018).

Another compound with ethereal notes is acetaldehyde that can be degraded by different LAB species. It has been shown that some LAB isolated from dairy matrices can metabolize it, producing ethanol and acetic acid as final products. It is therefore conceivable that the decrease observed in this work after fermentation with *L. rhamnosus* 1473 may be due to bacterial metabolism (Osborne et al., 2000). As can be seen in Table 3, the concentration of ethanol increased, but in this case it was recovered only in the distillate obtained under vacuum. Thanks to the unique enzymatic portfolio of LAB, they can activate different metabolic pathways to adapt to different environments. The activation of different metabolisms can lead to the catabolism of aldehydes, the synthesis and hydrolysis of esters, the degradation of phenolic acids, lipolysis, proteolysis, and peptidolysis (Hadj Saadoun et al, 2021). Moreover, different enzymes such as glycosidases, β-glucosidases, esterases, phenolic acid decarboxylases potentially produced by LAB possess an important role in the production of aromatic compounds (Cappello et al ., 2017).

Conclusions

The purpose of this work was to assess the ability of *L. rhamnosus* 1473, a LAB strain with recognized aromatic potential, to grow on fruit by-products to recover, from the fermented matrix, the aromatic fraction. Qualitative and quantitative comparison of the aromatic compounds obtained before and after fermentation, and by the use of two distillation

methods, was made to define the most promising strategy to be investigated in future studies.

In general, fermentation has led to interesting changes, increasing typical citrus, orange, and melon compounds. Esters and terpenes increased especially under fermentation, with particular attention for β-linalool and trans geraniol in orange by-products and 2-hexenal, α-terpineol and butanoic acid ethyl and methyl esters, as well as linalool or β-damascenone in melon by-products, compounds which could be employed in food or perfume industries.

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Appendix A. Supplementary data

Supplementary Table A1. Assignment of GC-MS signals of orange pomace distillates obtained after simple distillation.

Peak no.	Identification	LRI	Identification Method	Reference
1	Dimethyl sulfide	737	MS+LRI	Goodner, 2008
2	Acetone	801	MS+LRI	Tatsuka et al., 1990
3	Butanal,3-methyl-	877	MS+LRI	Qian & Reineccius., 2003
4	Acetic acid ethenyl ester	962	MS+LRI	Goodner, 2008
5	1R-α-Pinene	1013	MS+LRI	Goodner, 2008
6	3-buten-2-ol,e-methyl	1032	MS+LRI	Vinogradov , 2004
7	Hexanal	1076	MS+LRI	Goodner, 2008
8	β-pinene	1158	MS+LRI	Högnadóttir et al; 2003
9	Heptanal	1184	MS+LRI	Goodner, 2008
10	D-limonene	1197	MS+LRI	Goodner, 2008
11	cosmene	1212	MS+LRI	Gauvin et al., 2004
12	2-Hexanal-(E)	1148	MS+LRI	Goodner, 2008
13	teta Terpinene	1240	MS+LRI	Goodner, 2008
14	o-cymene	1245	MS+LRI	Gauvin et al., 2004
15	Octanal	1285	MS+LRI	Goodner, 2008
16	NI	1313		
17	Prenol	1316	MS+LRI	Umano et al., 1994
18	Sulcatone	1333	MS+LRI	Tressl et al., 1978
19	(cis)-Rose oxide	1346	MS+LRI	Ong et al., 1999
20	1-Hexanol	1346	MS+LRI	Goodner, 2008
21	(trans)-Rose oxide	1360	MS+LRI	NIST
22	Methyl Octanoate	1384	MS+LRI	Tressl et al., 1978
23	1,3,8-p-Menthatriene	1388	MS+LRI	Orav et al., 2003
24	β-isophorone	1402	MS+LRI	Le Guen et al., 2000
25	2-p-Tolylpropene	1433	MS+LRI	
26	1- Heptanol	1448	MS+LRI	Cirlini et al., 2012
27	NI	1452		
28	NI	1459		
29	Furfural	1463	MS+LRI	Pozo-Bayon et al., 2007
30	alfa copaene	1482	MS+LRI	Loayza et al., 1995
31	Decanal	1493	MS+LRI	Goodner, 2008
32	2-Acetylfuran	1502	MS+LRI	NIST
33	Ethanone,1-(1,4-dimethyl-3-cyclohexen-1-yl)	1514	MS	
34	Benzaldehyde	1520	MS+LRI	Bianchi et al., 2007
35	2-Nonenal,(E)-	1531	MS+LRI	
36	β-Linalool	1542	MS+LRI	Goodner, 2008
37	1-octanol	1551	MS+LRI	Goodner, 2008
38	cyclohexene, 2-ethenyl-1,3,3-trimetyl	1554	MS	

39	5-Methylfurfural	1572	MS+LRI	Priestap et al., 2003
40	beta-gurjunene	1582	MS+LRI	NIST
41	(-)-4-terpineol	1596	MS+LRI	
42	dihydrocarvone	1603	MS+LRI	NIST
43	p-Menth-1-en-9	1610	MS+LRI	NIST
45	β-Cyclocitral	1615	MS+LRI	NIST
46	NI	1622		
47	NI	1623		
48	Safranal	1639	MS+LRI	Kaypak et al., 2008
49	1-Nonanol	1652	MS+LRI	Goodner, 2008
50	β- Farnesene	1659	MS+LRI	
51	Cis-p-Mentha-2,8-dien-1-ol	1664	MS+LRI	Kollmannsberger et al., 1992
52	hexanoic acid,3-hydroxy-,ethyl ester	1673	MS+LRI	NIST
53	Citral	1680	MS+LRI	
54	p-Mentha-3-ene-8-ol	1690	MS+LRI	Goodner, 2008
55	Dodecanal	1703	MS+LRI	
56	valencene	1712	MS+LRI	
57	Selinene	1716	MS+LRI	
58	eucarvone	1724	MS+LRI	de la Fuente et al., 2005
59	Carvone	1729	MS+LRI	
60	2-Cyclopentyl Cyclopentanone	1741	MS	
62	1-Decanol	1754	MS+LRI	Goodner, 2008
63	Citronellyl Formate	1758	MS+LRI	Ferreira et al., 2001
64	Monomethyl succinate	1764	MS	
65	Ethanone, 1-(4-methylphenyl)-	1771	MS+LRI	Radovic et al., 2001
66	Perillaldehyde	1779	MS+LRI	
67	(+)-trans-carveol	1789	MS+LRI	
68	cis-Geraniol	1791	MS+LRI	Ricci A et al., 2018
69	trans-Chrysanthenyl acetate	1798	MS+LRI	Pino et al., 2001
70	NI	1805		
71	(+)-cis-carveol	1828	MS+LRI	
72	trans-geraniol	1838	MS+LRI	Ricci et al., 2018
73	Geranyl acetone	1847	MS+LRI	Buttery, 1982
74	(-)-cis-Carveol	1858	MS+LRI	Kollamannsberger, 1992
75	(-)-trans-Carveol	1880	MS+LRI	
76	Caryophyllene oxide	1974	MS+LRI	
77	Perillic alcohol	1987	MS+LRI	
78	NI	2010		
79	NI	2014		
80	Nerolidol	2027	MS+LRI	
81	Ethanone, 1-(2-hydroxy-5-methylphenyl)-	2176	MS+LRI	NIST
82	β-SINENSAL	2201	MS+LRI	Berlinet et al.,2005

NI: not identified; MS: mass spectrometer

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Supplementary Table A2. Assignment of GC-MS signals of orange pomace distillates obtained after vacuum distillation

Peak no.	Identification	LRI	Identification Method	Reference
1	Propane,2-methoxy,-2-methyl	680	MS	
2	Acetone	902	MS+LRI	Tatsuka et al., 1990
3	Ethyl Acetate	845	MS+LRI	Dall'Asta et al., 2011
4	Hetanol	891	MS+LRI	Ricci et al., 2018
5	3-Buten-2-ol,2-methyl-	1032	MS+LRI	
6	Hexanal	1076	MS+LRI	Goodner, 2008
7	NI	1125		
8	m-Xylene	1135	MS+LRI	Riganakos et al., 1999
9	NI	1167		
10	α-Terpinene	1172	MS+LRI	Bhattacharya, 1998
11	NI	1175		
12	Heptanal	1183	MS+LRI	Goodner, 2008
13	Limonene	1194	MS+LRI	Goodner, 2008
14	1-Butanol, 3-methyl-	1204	MS+LRI	Zarazir et al., 1970
15	Cosmene	1212	MS	
16	teta Terpinene	1240	MS+LRI	Goodner, 2008
17	o-cymene	1244	MS+LRI	Gauvin et al., 2004
18	Benzene, 1-dimethyl 3-(1-methylethyl)-	1265	MS	
19	Octanal	1286	MS+LRI	Goodner, 2008
20	Acetoin	1310	MS+LRI	Ricci et al., 2018
21	1-Hexanol	1346	MS+LRI	Goodner, 2008
22	Diacetone alcohol	1358	MS+LRI	Umano et al., 1999
23	1,3,8-p-Menthatriene	1388	MS+LRI	Orav et al., 2003
24	2-p-Tolylpropene	1433	MS+LRI	Choi, 2003b.
25	Limonene oxide, trans	1451	MS+LRI	
26	Furfural	1463	MS+LRI	Pozo-Bayon et al., 2007
27	1-Hexanol,2-ethyl	1482	MS+LRI	NIST
28	Decanal	1492	MS+LRI	Goodner, 2008
29	NI	1500		
30	NI	1516		
31	Benzaldehyde	1520	MS+LRI	Bianchi et al., 2007
32	beta Linalool	1540	MS+LRI	Cirlini et al., 2012
33	1-Octanol	1549	MS+LRI	Goodner, 2008
34	cis-p-menth-2-en-1-ol	1554	MS+LRI	
35	Lilac aldehyde	1558	MS+LRI	NIST
36	2-Furaldehyde,5-methyl	1571	MS+LRI	NIST
37	NI	1573		
38	4-terpineol	1594	MS+LRI	NIST
39	p-Menth-8-en-2-one	1602	MS+LRI	
40	1 H-Pyrrole-2-carboxaldehyde, 1-ethyl	1605	MS+LRI	NIST
41	methyl benzoate	1617	MS+LRI	Ferreira et al., 2001

42	trans-p-menth-2-en-1-ol	1620	MS+LRI	Avato et al., 2004
43	NI	1623		
44	Menthol	1631	MS+LRI	
45	NI	1629		
46	Safranal	1639	MS+LRI	Kaypak et al., 2008
47	1-Nonanol	1650	MS+LRI	Goodner, 2008
48	3-Furanmethanol	1657	MS+LRI	Alasalvar et al., 2003
49	cis-p-mentha-2,8-dien-1-ol	1662	MS+LRI	Kollmannsberger et al., 1992
50	Hexanoic acid, 3-hydroxy-, ethyl ester	1673	MS+LRI	NIST
51	Citral	1679	MS+LRI	
52	alfa-Terpineol	1689	MS+LRI	
53	Butanethioic acid, 3-oxo-, S-ethyl ester	1700	MS	
54	Valencene	1707	MS+LRI	
55	4-Methyleneisophorone	1723	MS	
56	Carvone	1727	MS+LRI	Goodner, 2008
57	1,5,7-Octatrien-3-ol,2,6-dimethyl	1738	MS	
58	(-)-cis-Isopiperitenol	1740	MS+LRI	
59	(-)-trans-Isopiperitenol	1745	MS+LRI	NIST
60	1-Decanol	1752	MS+LRI	Goodner, 2008
61	Citronellol	1756	MS+LRI	Ferreira et al., 2001
62	NI	1760		
63	trans-p-mentha-1(7),8-dien-2-ol	1788	MS+LRI	NIST
64	Nerol	1790	MS+LRI	Chisholm et al., 2003
65	Hexane, 1-chloro-5-methyl	1798	MS	
66	Vetivenene	1803	MS	
67	(-)-cis-Carveol	1827	MS+LRI	
68	isopiperitenone	1833	MS	
69	Geraniol	1837	MS+LRI	Goodner, 2008
70	m-Cymen-8-ol	1842	MS+LRI	
71	5,9-Undecadien-2-one,6,10-dimethyl-,E	1846	MS+LRI	Buttery et al., 1982
72	Carveol	1857	MS+LRI	
73	2,2,4-Trimethyl-1,3-pentanediol diisobutyrate	1866	MS	
74	Benzyl alcohol	1871	MS+LRI	Goodner, 2008
75	cis-p-mentha-1-(7),8-dien-2-ol	1880	MS	
76	Hexanoic acid, 2-ethoxethyl ester	1886	MS	
77	Butylated Hydroxytoluene	1904	MS+LRI	
78	NI	1905	MS	
79	Piperitenone	1915	MS+LRI	
80	1- Dodecanol	1955	MS+LRI	NIST
81	Biphenyl	1982	MS+LRI	
82	p-Mentha-1(7),8(10)-dien-9-ol	1987	MS+LRI	
83	p-Mentha-1,8-dien-7-ol	1996	MS+LRI	Umano et al., 2002

84	NI	2010	MS	
85	Isopropyl myristate	2021	MS+LRI	NIST
86	Nerolidol trans-	2027	MS+LRI	
87	Epicubenol	2050	MS+LRI	
88	4,6-Dioxadodecane	2052	MS	
89	3,7-Cyclodecadiene-1-methanol, alfa,alfa A,8-tetramethyl-,[s-(Z,Z)]	2064	MS+LRI	NIST
90	2,5-cyclohexadien-1-one,2,6-bis(1,1-dimethylethyl)-4-hydroxy-4-methyl	2083	MS+LRI	Miyazawa et al., 2003
91	Spathulenol	2105	MS+LRI	
92	gamma-Eudesmol	2144	MS+LRI	
93	Eugenol	2149	MS+LRI	Ricci et al., 2018
94	Pogostole	2157	MS	
95	2-Menthoxy-4-vinyphenol	2176	MS+LRI	Cadahia et al., 2003
96	Hexadecanoic acid, methyl ester	2185	MS+LRI	Pino et al., 2001
97	gamma-Eudesmol	2200	MS+LRI	Berlinet et al., 2005

NI: not identified; MS: mass spectrometer

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Supplementary Table A3. Assignment of GC-MS signals of orange pulp distillates obtained after simple distillation.

Peak no.	Identification	LRI	Identification Method	Reference
1	Acetaldehyde	705	MS+LRI	Goodner, 2008
2	Ethyl Acetate	846	MS+LRI	Goodner, 2008
3	Isovaleraldeide	877	MS+LRI	Qian & Reineccius, 2003
4	Ethanol	892	MS+LRI	Goodner, 2008
5	Butanoic acid, methyl ester	874	MS+LRI	Ulrich et al., 1997
6	Butanoic acid, 2-methyl-,methyl ester	1006	MS+LRI	NIST
7	Butanoic acid, ethyl ester	1030	MS+LRI	Chastrette et al., 1974
9	Furan, 2,3-dihydro-	1038	MS	
10	Butanoic acid, 2-methyl-ethyl ester	1044	MS+LRI	NIST
11	Disulfide dimethyl	1065	MS+LRI	Shibamoto et al., 1981
12	Hexanal	1074	MS+LRI	Goodner, 2008
13	Isobutanol	1092	MS+LRI	
14	1-Butanol	1141	MS+LRI	Goodner, 2008
15	2-Pentenal, 2-methyl	1148	MS+LRI	NIST
16	Butanoic acid, 2-methyl-,2-methylpropyl ester	1170	MS+LRI	Boulanger et al., 2000
17	Heptanal	1183	MS+LRI	Goodner, 2008
18	Eucalyptol	1201	MS+LRI	
19	NI	1194		
20	1-Butanol, 2-methyl	1205	MS+LRI	Lee et al., 2003
21	2-Hexenal, E	1218	MS+LRI	
22	ethyl hexanoate	1230	MS+LRI	Lee et al., 2003
23	1-Pentanol	1246	MS+LRI	Goodner, 2008
24	Acetic acid, hexyl ester	1268	MS+LRI	Spencer et al., 1978
25	Butanoic acid, 2-methyl-2-methylbutyl ester	1274	MS+LRI	Liu et al., 2001
26	Octanal	1285	MS+LRI	Goodner, 2008
27	3-Hexenoic acid, ethyl ester	1300	MS+LRI	NIST
28	Cyclohexanone,2,2,6-trimethyl	1310	MS+LRI	Kawakami et al., 1993
29	NI	1317		
30	2-Hexenal, 2-ethyl	1329	MS+LRI	Choi et al., 2002
31	Sulcatone	1333	MS+LRI	Tressl et al., 1978
32	1-Hexanol	1347	MS+LRI	Goodner, 2008
33	Dimethyl trisulfide	1374	MS+LRI	Kim et al., 2004
34	3-Hexen-1-ol, (Z)	1378	MS+LRI	
35	Nonanal	1388	MS+LRI	Goodner, 2008
36	Hexanoic acid, butyl ester	1405	MS+LRI	Chastrette et al., 1974
37	Hexanoic acid, hexyl ester	1408	MS	
38	2-Octenal E	1425	MS+LRI	Högnadóttir et al., 2003
39	Octanoic acid, ethyl ester	1428	MS+LRI	Riu-Aumatell et al., 2004
40	1-octen-3-ol	1443	MS+LRI	Karlsson et al., 2009
41	1-Heptonol	1448	MS+LRI	de la Fuente et al., 2005
42	Isopenthyl hexanoate	1452	MS+LRI	
43	3-Furaldehyde	1463	MS+LRI	Golovnya et al., 1992

44	Acetic acid, octyl ester	1468	MS+LRI	Goodner, 2008
45	Ethyl Sorbate	1478	MS+LRI	Pino et al.,2001
46	2-Ethyl-1-hexanol	1482	MS+LRI	Romeo et al., 2009
47	Decanal	1492	MS+LRI	Goodner, 2008
48	3-Octen-1-ol,acetate, Z	1501	MS+LRI	Kourkoutas et al., 2006
49	Benzaldehyde	1522	MS+LRI	Bianchi et al., 2007
50	2-Nonenal	1531	MS+LRI	NIST
51	beta-Linalool	1541	MS+LRI	Goodner, 2008
52	1-Octanol	1551	MS+LRI	
53	3-Octen-1-ol	1576	MS+LRI	NIST
54	2,6-Nonadienal	1582	MS+LRI	Ferreira et al., 2001
55	Isocaryophylene	1587	MS+LRI	Tabanca et al., 2005
56	Undecanal	1597	MS+LRI	Bais et al., 2003
57	beta-cyclocitral	1614	MS+LRI	NIST
58	NI	1624		
59	NI	1629		
60	Decanoic acid, ethyl ester	1630	MS+LRI	Riu-Aumatell et al., 2006
61	2-Decenal,E	1638	MS+LRI	Lin et al., 2001
62	1-Nonanol	1651	MS+LRI	Goodner, 2008
63	3-Nonen-1-ol	1675	MS+LRI	
64	Ethyl trans-4-decenoate	1684	MS+LRI	NIST
65	alfa terpineol	1689	MS+LRI	Goodner, 2008
66	3-Decen-1-ol,acetate	1696	MS+LRI	Vinogradov.,2004
67	cis-6-Nonen-1-ol	1707	MS+LRI	
68	alfa Muurolene	1713	MS+LRI	
69	NI	1718		
70	3,6 Nonadien -1-ol	1742	MS	
71	beta- Cadinene	1746	MS+LRI	NIST
72	cis-4-Decen-1-ol	1751	MS+LRI	Werkhoff et al., 1998
73	NI	1758		
74	3-Decen-1-ol	1776	MS+LRI	
75	Alfa Cadinene	1781	MS+LRI	
76	NI	1802		
77	beta- Damascenone	1813	MS+LRI	Pontes et al., 2007
78	Ethyl laurate	1833	MS+LRI	
79	5,9-Undecadien-2-one, 6,10-dimethyl,-E	1847	MS+LRI	Buttery et al., 1982
80	Benzyl alcohol	1871	MS+LRI	Goodner, 2008
81	Phenylethyl Alcohol	1905	MS+LRI	
82	alfa Ionone	1930	MS+LRI	
83	Dihydro-β-ionone	1940	MS+LRI	Carrer et al., 2007
84	5,9-Undecadien-2-ol, 6,10-dimethyl-	1944	MS+LRI	Joichi et al., 2005
85	Myristoleyl alcohol	1979	MS	
86	8-Dodecen-1-ol,Z	1994	MS+LRI	Marques et al., 2000
87	10-Undecyn-1-ol	2027	MS+LRI	
88	3-Phenylpropanol	2036	MS+LRI	Osorio et al., 2006
89	isoaromadendrene epoxide	2057	MS	
90	cis,cis,cis-7,10,13-Hexadecatrienal	2090	MS	
91	.tau.-Cadinol	2161	MS+LRI	

92	.tau.-Muurolol	2161	MS+LRI	Gaydou et al., 1986
93	delta-cadinol	2172	MS+LRI	Tressl et al., 1978
94	2-Methoxy-4-vinylphenol	2177	MS+LRI	Cadahia et al., 2003
95	alfa-Cadinol	2201	MS+LRI	
96	Methyl palmitoleate	2210	MS+LRI	NIST
97	Hexadecanoic acid, ethyl ester	2218	MS+LRI	Brat et al., 2003
98	Ethyl 9-hexadecenoate	2241	MS+LRI	
99	2,4-Di-tert-butyphenol	2274	MS+LRI	

NI: not identified; MS: mass spectrometer

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Supplementary Table A4. Assignment of GC-MS signals of melon pulp distillates obtained after vacuum distillation.

Peak no.	Identification	LRI	Identification Method	Reference
1	Acetaldehyde	705	MS+LRI	Goodner et al., 2008
2	Acetone	801	MS+LRI	Tatsuka et al.; 1990
3	Ethyl Acetate	847	MS+LRI	Goodner et al., 2008
5	Ethanol	892	MS+LRI	Goodner et al., 2008
6	Butanoic acid, ethyl ester	1030	MS+LRI	Chastrette et al., 1974
7	Butanoic acid,2-methyl-, ethyl ester	1043	MS+LRI	NIST
8	Hexanal	1076	MS+LRI	Goodner et al., 2008
9	Isobutanol	1085	MS+LRI	Tressl et al., 1978
10	2-Butenal, 2-methyl	1096	MS+LRI	Calvo-Gomez et al., 2004
11	NI	1124		
12	1-Butanol	1139	MS+LRI	Goodner et al., 2008
13	NI	1184		
14	D-Limonene	1193	MS+LRI	Mallavarapu et al., 1998
15	1-Butanol, 2-methyl	1204	MS+LRI	Aubert et al., 2004
16	NI	1223		
17	NI	1240		
18	1-Pentanol	1246	MS+LRI	Goodner et al., 2008
19	Acetoin	1285	MS+LRI	
20	NI	1315		
21	1-Hexanol	1346	MS+LRI	Goodner et al., 2008
22	Diacetone alcohol	1358	MS+LRI	
23	3-Hexen-1-ol,(Z)	1378	MS+LRI	
24	Nonanal	1388	MS+LRI	Goodner et al., 2008
25	NI	1394		
26	Methyl 2- (methylthio)acetate	1405	MS+LRI	Aubert et al., 2004
27	1-Octen-3-ol	1443	MS+LRI	Karlsson et al., 2009
28	1-Heptanol	1448	MS+LRI	de la Fuente et al., 2005
29	Propanal,3-(methythio)	1453	MS+LRI	Chen et al., 2009
30	Furfural	1463	MS+LRI	Schwambach et al., 2006
31	2-Ethyl-1-hexanol	1482	MS+LRI	Romeo et al., 2009
32	Decanal	1492	MS+LRI	Goodner et al., 2008
33	Butanoic acid, 3-hydroxy-, ethyl ester	1513	MS+LRI	Morales et al., 2000
34	Benzaldehyde	1520	MS+LRI	Bianchi et al., 2007
35	2-Nonenal,E	1530	MS+LRI	NIST
36	beta-Linalool	1540	MS+LRI	Goodner et al., 2008
37	1-Octanol	1549	MS+LRI	Prososki et al., 2007
38	3-Octen-1-ol	1576	MS	
39	2,6-Nonadienal,E,Z	1581	MS+LRI	Ferreira et al., 2001
40	NI	1594		
41	Ethanone, 1-(2-pyridinyl)	1598	MS+LRI	Viegas et al., 2007
42	5-Octen-1-ol,Z	1608	MS+LRI	NIST

43	Megastigma-4-6,(Z),8(Z)-triene	1613	MS+LRI	NIST
44	Decanoic acid, ethyl ester	1630	MS+LRI	Riu-Aumatell et al., 2006
45	Benzeneacetic acid, 2-hexenyl ester	1641	MS+LRI	NIST
46	1-Nonanol	1650	MS+LRI	Goodner et al., 2008
47	3-Nonen-1-ol	1675	MS+LRI	Hayata et al., 2003
48	alfa Terpineol	1688	MS+LRI	Goodner et al., 2008
49	NI	1699		
50	6-nonenol	1708	MS+LRI	Pennarun et al., 2003
51	3,6-Nonadien-1-ol	1742	MS+LRI	Pennarun et al., 2003
52	beta cadinene	1746	MS+LRI	NIST
53	cis-4-Decen-1-ol	1750	MS+LRI	Werkhoff et al., 1998
54	1-Decanol	1752	MS+LRI	
55	3-Decen,1-ol	1775	MS+LRI	Tamura et al., 1991
56	5-Decen-1-ol	1798	MS+LRI	Tamura et al., 1990
57	NI	1802		
58	beta- Damascenone	1813	MS+LRI	Pontes et al., 2007
59	Ethyl laurate	1832	MS+LRI	
60	trans-Geraniol	1838	MS+LRI	Miyazawa et al., 2003
61	5,9-Undecadien-1-one, 6,10-dimethyl	1846	MS+LRI	Buttery et al., 1982
62	NI	1851		
63	Benzyl Alcohol	1871	MS+LRI	Goodner et al., 2008
64	Phenylethyl Alcohol	1905	MS+LRI	Komes et al., 2006
65	2-Phenyl-2-butenal	1926	MS+LRI	Mahadevan et al., 2006
66	trans-Beta-Ionone	1930	MS+LRI	
67	1-Dodecanol	1956	MS+LRI	Ferrari et al., 2004
68	cis-7-Tetradecen-1-ol	1978	MS+LRI	NIST
69	Isopropyl myristate	2022	MS+LRI	Tsakiris et al., 2004
70	3-Phenylpropanol	2036	MS+LRI	NIST
71	cis,cis,cis-7,10,13-Hexadecatrienal	2090	MS	
72	delta-Cadinol	2147	MS+LRI	
73	Eugenol	2149	MS+LRI	Ricci et al., 2018
74	tau-Cadinol	2161	MS+LRI	
75	2-Methoxy-4-vinylphenol	2177	MS+LRI	Cadahia et al., 2003
76	alfa-Cadinol	2200	MS+LRI	
77	Methyl palmitoleate	2209	MS+LRI	NIST
78	Hexadecanoic acid, ethyl ester	2217	MS+LRI	Brat et al., 2003
79	Ethyl 9-hexadecenoate	2240	MS+LRI	

NI: not identified; MS: mass spectrometer

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Chapter 2.2

Effect of fermentation with single and co-culture of lactic acid bacteria on okara evaluation of bioactive compounds and volatile profiles

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Abstract

Okara is the main soybean by-product deriving from the processing of soy milk and tofu. Despite being a product with a lot of potential, rich in many bioactive compounds such as polyphenols, it presents an unpleasant, rancid aroma. For these reasons its use in food industry is limited. In this study, we reported the integral use of okara in a solid state fermentation process, conducted with wild strains of lactic acid bacteria, to evaluate the effect of bacterial metabolism on volatile and polyphenolic profile. Strains belonging to *Lactobacillus acidophilus*, *Lacticaseibacillus rhamnosus* and *Pediococcus acidilactici* species were used in mono-culture and, for the first time, in co-culture. Results showed an improvement in the aromatic fraction showing a decrease of hexanal, responsible for off-flavour, and an increase of ketones with fruity and buttery notes in fermented okara. Polyphenols were also affected, and, in particular, a bioconversion of glucoside isoflavones to the aglycone forms was highlighted in all fermented substrates. In addition, a release of phenyllactic, hydroxyphenyllactic and indole-3-lactic acids was observed for the first time. Overall, the co-culture appears the most promising for biovalorization of okara opening the possibility of its use in the development of functional ingredients.

Introduction

Okara is the by-product resulting from the production of soy milk and tofu, after filtration of crushed soybeans. Following the increase in the demand for soy-based products in Europe and habitual consumption in Asia, large quantities of okara are produced every year. The high production of okara currently represents a significant disposal problem for both industry and the environment, in fact each kilogram of dry soybeans generates about 1.1 kg of okara (Vong & Liu, 2016). This by-product is mainly used in the feed sector or discarded, although it is still rich in high quality proteins, unsaturated fatty acids, dietary fiber, isoflavones, minerals and oligosaccharides (Vital et al., 2018). The two main isoflavone glycosides, genistin and daidzin, are present in soybean in the form of β-D-glycoside. Some studies demonstrated that the corresponding aglycones genistein and daidzein, released by the action of β-glucosidase, exhibited higher biological activity and suggested that these aglycones can be better absorbed upon consumption, possibly because of the lower molecular weight and lower hydrophilicity (Queiroz Santos et al., 2018; Y. Zhu et al., 2019). Some of the main drawbacks in the valorization of okara are its high degree of perishability, the presence of compounds with anti-nutritional effects and undesirable off-flavors and rancid aromas, caused by the oxidation of polyunsaturated lipids by the enzyme lipoxygenase, present in soybeans (Queiroz Santos et al., 2018).

Solid state fermentation (SSF) has been used, in last years, as a strategy to add value to okara. SSF is defined as a bioprocess where microbial growth and product formation occur on the surface of solid materials, almost in the absence of free water. Considering the limited amount of water and the water activity values, only fungi and yeast should be suitable for this process, but also specific bacterial cultures can be employed, showing good performances (Pandey, 2003). In this context, Lactic Acid Bacteria (LAB), generally used as starter cultures to drive food fermentations, were recently used for the SSF processes of waste and by-products (Ahmad et al., 2020; Immonen et al., 2020). Fermentation has recently been applied to improve the flavor and texture of okara for food applications (Marsilvio Lima de Moraes Filho et al., 2018) but also to enhance the health attributes, ideally through the production of functional ingredients (Chan et al., 2019). Differently from these studies,

in this work, we proposed the integral use of okara, without pre-treatments or additives, in a solid state fermentation process conducted with LAB. As studies regarding the use of only probiotic strain of LAB (Shi et al., 2020; Voss et al., 2018) are present in the literature, we investigated the use of wild strains, isolated from different niches, belonging to *Lactobacillus acidophilus*, *Lacticaseibacillus rhamnosus* and *Pediococcus acidilactici.* These species are reported to grow on soy and okara (Shi et al., 2020; Voss et al., 2018) or to reduce the beany-flavor (Blagden & Gilliland, 2006; Schindler et al., 2011). Considering that LAB-LAB co-cultures have not been widely studied, although they seem advantageous compared to single cultures due to the synergistic action of the metabolic pathways of the strains involved (Chen, 2011), we carried out a comparison between mono and co-culture to define the best conditions to improve phytochemical and aromatic features of okara.

Materials and methods

Chemicals

Toluene used as reference for HS-SPME/GC-MS analyses was obtained from Sigma-Aldrich, USA. Phenyllactic acid, indole-3-lactic acid and genistein were purchased from Sigma-Aldrich (St. Louis, MO, USA). Daidzein was from AASC Ltd. (Southampton, UK) while *p*-hydroxyphenyllactic acid from Santa Cruz Biotechnology (Dallas, TX, USA). Both HPLC-grade water and HPLC-grade acetonitrile were purchased from VWR International (Milan, Italy), as well as methanol and LC-MS grade formic acid.

Okara fermentation

The okara used for this work was provided by Sojasun company located in Fidenza (Parma, Italy) and stored at -80 °C to avoid deterioration, due to the high activity of water. Before fermentation, the substrate was autoclaved at 121 °C for 20 minutes.

The starter inoculum was prepared cultivating the revitalized strains until the late exponential phase (ca. 15 h), harvesting the cell by centrifugation (12,857× g for 10 min at 4 °C), washing twice with Ringer's solution (Oxoid, Milan, Italy), and finally re-suspending in sterile distilled water to a final concentration of 9.0 Log CFU/mL. Each culture was inoculated into 30 g of okara to reach 6 -7 Log CFU/g. The inoculum was homogenized in

the sample by mixing for 2 minutes with a sterile loop. Co-culture was obtained by mixing single revitalized strains in equal volume and further diluting the mixture to reach 6-7 Log CFU/g in the product. The okara was fermented at 37 °C with all the strains and co-culture for 72 h.

Each fermentation was performed in duplicate. Samples were analyzed after inoculum (T_0) and at the end of fermentation process (T_{72}) by viable cell counts, carried out by plate count on MRS agar (Oxoid, Milan, Italy), incubating at 37 °C for 48 h.

Investigation of the volatile composition of fermented okara by HS-SPME/GC-MS technique

The volatile profile of fermented and unfermented okara was analyzed by HS-SPME/GC-MS technique following the protocol reported by Ricci, Cirlini, et al. (2018) with slight modifications. In particular, 3 g of okara and 10 μL of an aqueous toluene standard solution (100 μg/mL in 10 mL) were used for the analyses.

GC–MS analyses were performed on a Thermo Scientific Trace 1300 gas chromatograph interfaced with a Thermo Scientific ISQ single quadrupole mass spectrometer, equipped with an electronic impact (EI) source (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The column used for the analytes separation, as GC and MS parameters, HS-SPME sampling conditions in terms of equilibration and extraction time and temperature, and fiber type were the same described Ricci, Cirlini, et al. (2018). After the analyses, the gas-chromatographic detected signals were identified by both the comparison of the obtained mass spectra with those present in the instrument library (NIST-14), as by calculation of their linear retention indexes (LRIs). In addition, the semi-quantification of the identified compounds was performed based on a reference (Toluene).

Extraction of polyphenolic compounds and LAB-derived metabolites

To carry out the extraction of polyphenols, fermented and unfermented samples were firstly freeze-dried with a lyophilizer (Lio 5PDGT, Cinquepascal, Italy). The samples were subjected to the extraction of free polyphenols, in particular 3 mL of a methanol/water solution (80:20 v/v) acidified with 0.2% of formic acid were added to 0.3 g of each sample.

All samples were stirred for 1 min with vortex and 10 min shaker then treated for 15 min in an ultrasonic bath, other 10 min in a shaker and finally centrifuged for 25 min at 12,857× g, 4 °C. The supernatant was collected while the pellet obtained was re-suspended in 0.3 mL of methanol-water solution and subjected to a second and consequently to a third extraction, performed as described before. The supernatants were pooled and then diluted with a solution of water/methanol (80:20 v/v) acidified with 0.1% formic acid, then centrifuged at 14,462 g for 5 min before UHPLC-MS^n analyses.

Determination of polyphenolic profile and LAB-derived metabolites through UHPLC MS^n

The samples were analyzed with an Accela UHPLC 1250 interfaced with an ion trap mass spectrometer (LTQ-MS) (LTQ XL, Thermo Fisher Scientific Inc., San Jose, CA, USA) equipped with an ESI interface (H-ESI-II). Chromatographic separation was carried out through an Acquity UPLC HSS T3 column (2.1X 100 mm), 1.8 μm particle size equipped with an Acquity UPLC HSS T3 VanGuard pre-column (2.1 x 5 mm) was used (Water, Ireland). The chromatographic and mass spectrometer conditions were the same reported by Ricci, Cirlini, Calani, et al. (2019). Analyses of okara samples were carried out in negative ionization mode using full-scan, data-dependant MS^3 scanning from m/z 100 to 2000. Phenyllactic acid, indole-3-lactic acid, *p*-hydroxyphenyllactic acid, genistein and daidzein aglycones were quantified with their authentic standard compounds by extracting the corresponding deprotonated molecule ($[M-H]^-$) in the full scan chromatograms. Calibration curves of phenyllactic, indole-3-lactic and *p*-hydroxyphenyllactic acids ranged from 0.5 to 50 μmol/L, while calibration curves of both genistein and daidzein ranged from 0.05 to 20 μmol/L. Instead, the *O*-glycosylated isoflavones, especially the *O*-acetylglycosides, showed a very high fragmentation behavior in the negative ESI source, leading thus inadequate monitoring of their corresponding $[M-H]^-$ to avoid a loss of sensitivity. Thus, all *O*-glycosylated daidzein and genistein at each retention time were quantified by extracting the corresponding ion of daidzein and genistein at m/z 253 and 269, respectively. Glycitein aglycone and glycitein-*O*-glycoside were quantified as genistein equivalent by using the

same approach reported for genistein and daidzein glycosides. The identification of compounds listed in Table 3 was performed by comparison of MS^n ion spectra with the MS^n data stored in several online libraries as PubChem (https://pubchem.ncbi.nlm.nih.gov/); mzCloud (www.mzcloud.org/home); Metlin (http://metlin.scripps.edu); MoNA – Mass Bank of North America (https://mona.fiehnlab.ucdavis.edu/). Additional MS^n information was obtained through previous works (Axel et al., 2016; Kang et al., 2007).

Statistical analyses

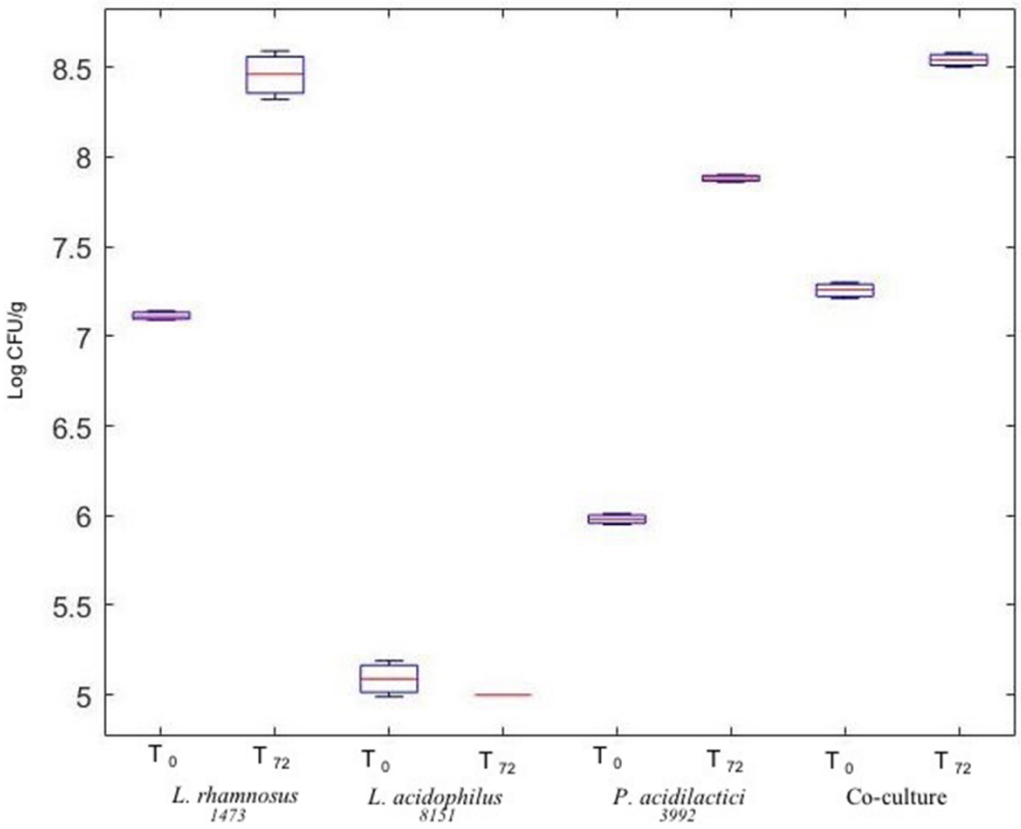
The data obtained from the analysis of volatile and polyphenolic profile were analyzed using the analysis of variance (one way ANOVA) and significant differences among the means ($p < 0.05$) were determined applying Tukey post hoc test using SPSS 21.0 software (SPSS Inc., Chicago, IL, USA) for different samples. Heat map was carried out using Heatmapper (www.heatmapper.ca) while alluvial diagram using Rawgraph (https://rawgraphs.io/).

Results and discussion

Evaluation of lactic acid bacteria growth

SSF of okara was carried out by inoculating three different LAB strains, namely *L. acidophilus* (8151), *L. rhamnosus* (1473), *Pediococcus acidilactici* (3992), and their co-coculture, at the concentration of 6-7 Log CFU/g. The microbial growth ability was assessed immediately after inoculation (T_0) and after 72 h of fermentation (T_{72}) at 37 °C, by plate counting on MRS agar. Results (Figure 1) highlighted that not all the tested strains were able to grow.

Figure 1. Box plot representing viable cell concentration (Log CFU/g) of strains in okara after inoculum (T0) and after 72 hours (T72) of fermentation at 37° C



Differences in growth performance may be ascribed to the different adaptability of the strains in stressful matrices, such as integral okara without pre-treatment and nutrient addition. Contrary to the observation of Moraes et al. (2016), where okara was added only in low percentage to soymilk, the strains of *L. acidophilus* used in this work did not show growth, probably due to the different composition of substrate and nutrients. According to Pereira et al. (2011), the growth of microorganisms depends on various factors, such as the substrate used and the strain employed

In agreement with Voss et al., (2018), *L. rhamnosus* shows the ability to grow in okara samples, demonstrating as for *P. acidilactici,* a higher adaptability increase the microbial load of 1 Log cfu/g. Also in the case of co-culture, an increase of bacterial concentration was observed, probably due to a synergistic effect of strains. Interactions in LAB-LAB co-cultures in SSF process had never been studied, but the knowledge acquired in food industry shows that metabolic interactions among bacteria can be useful to modify the substrate. In particular, the use of co-cultures seems advantageous compared to the single culture, due to the synergistic action of the metabolic pathways of the strains involved

(Chen, 2011), leading to increased degradation of the substrates (Bader et al., 2010), with a consequent increase of peptides and amino acids (Gobbetti et al., 1994), organic acids (Settachaimongkon et al., 2014), and volatile compounds (Álvarez-Martín et al., 2008).

Volatile profile of fermented and unfermented okara

The characterization of the volatile composition of fermented and unfermented okara was performed by HS-SPME/GC-MS technique. A total of 42 different compounds, belonging to different classes (aldehydes, alcohols, ketones and furan compounds) were detected. The full identification of all detected volatile compounds is reported in Table S1.

Significant differences between fermented and unfermented samples were recorded mainly for aldehydes. A high concentration of aldehydes was observed both in the control (1450.64±296.01 μg/g) and in the sample inoculated with *L. acidophilus* (866.12±70.59 μg/g), while a decrease was recorded in okara fermented with *P. acidilactici* (282.92±37.79 μg/g), co-culture (68.28±5.86 μg/g) and *L. rhamnosus* (25.29±5.66 μg/g).

The compound responsible for these variations is mainly hexanal, with a persistent herbaceous aroma, generated by lipid oxidation (Wang & Cha, 2018), resulting in the unpleasant smell of the soybean-based products. Notably, a significant decrease of its concentration was observed (Table 1) after 72 hours in *P. acidilactici* fermented sample, while in *L. rhamnosus* and co-culture fermented samples this compound was undetected.

Table 1. Concentration (μg/g) of volatile compounds found in unfermented (control) and fermented okara with different strains for 72 hours

	Control	***L. acidophilus*** **8151**	***P. acidilactici*** **3992**	***L. rhamnosus*** **1473**	**Co-culture**
Aldehydes					
Pentanal	46.37±28.73[a]	29.08±5.93[a]	9.84±2.01[a]	ND	ND
Hexanal	494.83±8.57[a]	492.95±103.37[a]	147.64±38.95[b]	ND	ND
Heptanal	139.49±48.09[a]	76.79±31.43[a,b]	27.37±4.06[b]	ND	ND
Octanal	124.31±66.77[a]	35.18±2.96 [a]	19.66±3.89 [a]	ND	ND
2-Heptenal	127.74±10.71[a]	15.33±1.70[b]	6.35±3.39[b]	ND	9.21±1.10[b]

Nonanal	208.27±117.35[a]	49.64±11.51[a]	17.47±1.52[a]	ND	ND
2-Octenal (E)	95.87±46.06[a]	19.33±2.25[b]	8.84±1.93[b]	ND	12.91±3.88[b]
Furfural	23.66±7.87[a]	3.73±1.46[b]	ND	ND	ND
Decanal	46.73±21.40[a]	15.74±2.56[a]	ND	ND	ND
Benzaldehyde	114.58±51.76[a]	109.88±18.37[a]	44.96±4.09[a]	22.44±4.50[a]	46.16±3.31[a]
Dodecanal	5.83±2.98[a]	ND	ND	ND	ND
Benzaldehyde, 4-ethyl	9.95±3.52[a]	5.80±0.25[a]	1.80±0.11[b]	ND	ND
2,4-Decadienal	13.01±4.83[a]	12.67±1.62[a]	4.62±1.46[b]	ND	ND
Benzaldehyde, 2,5-dimethyl	ND	ND	ND	4.09±1.75[a]	ND
Total	1450.64±296.01[a]	866.12±70.59[a]	282.92±37.79[b]	25.29±5.66[b]	68.28±5.86[b]
Alcohols					
Propan-2-ol	131.81±10.15[a,b]	235.48±93.96[a]	86.83±7.90[a,b]	37.29±11.86[b]	65.44±20.80[b]
Ethanol	44.03±42.39[a]	57.44±13.89[a]	20.38±2.75[a]	13.76±1.37[a]	19.62±5.50[a]
1-Butanol,3-methyl	ND	7.64±2.29[a]	9.81±0.68[a]	2.09±0.15[b]	ND
1-Pentanol	90.17 ±50.94[a]	42.71±15.78[a]	18.90±1.55[a]	42.33±3.58[a]	ND
1-Hexanol	64.21±27.45[b]	206.74±46.22[a]	217.53±10.72[a]	216.96±25.33[a]	16.01±3.58[b]
3-Octanol	ND	ND	6.88±2.33[a]	8.08±0.01[a]	ND
1-Octen-3-ol	235.68±124.19[a]	225.55±67.58[a]	107.51±1.73[a]	101.03±1.89[a]	225.51±20.94[a]
1-Heptanol	33.33±16.08[a]	19.81±2.25[a]	15.56±1.34[a]	12.48±2.10[a]	ND
4-Ethylcyclohexanol	64.03±25.17[a]	0.84±0.34[c]	5.21±0.87[b]	8.92±2.53[b]	8.50±0.66[b]
1-Octanol	29.98±7.43[a]	34.67±7.54[a]	17.81±3.64[a,b]	11.67±3.90[b]	1.68±0.01[b]
2-Octen-1-ol	3.43± 2.72[b]	13.41±1.85[a]	3.60±0.62[b]	3.15±0.99[b]	5.86±0.67[b]
Benzyl Alcohol	5.57±1.97[a]	8.01±3.21[a]	5.26±1.70[a]	4.96±0.20[a]	6.27±1.56[a]
Phenylethyl Alcohol	1.11±1.09[a]	1.82±0.77[a]	ND	1.66±0.93[a]	2.06±0.73[a]
Total	666.46±167.67[a]	853.95±178.20[a]	636.94±115.19[a]	464.38±36.57[a]	365.63±4.67[a]
Ketones					
Acetone	45.09±20.99[a,b]	48.17±12.86[a,b]	18.75±0.09[b]	20.99±0.26[b]	84.39±4.69[a]

2-Butanone	1.35±0.16[a]	0.95±0.48[a]	ND	ND	ND
2-Heptanone	49.53±0.30[b]	29.55±5.56[b]	27.08±1.57[b]	36.21±1.15[b]	1581.64±61.19[a]
2-Octanone	1.49±1.18[a]	1.54±0.61[a]	1.16±0.61[a]	1.14±0.25[a]	3.81±0.99[a]
2-Butanone-3-hydroxy	ND	ND	ND	ND	166.30±3.62[a]
2-Nonanone	ND	ND	ND	ND	461.54±9.53[a]
3-Octen-2-one	13.93±2.88[a]	8.52±1.16[a,b]	5.01±2.11[b]	2.31±0.58[b]	18.70±0.97[a]
3,5-Octadien-2-one	4.72±2.60[a]	10.07±5.76[a]	5.84±1.39[a]	3.61±0.10[a]	11.05±2.80[a]
2-Undecanone	ND	ND	ND	ND	28.18±2.94[a]
Total	116.10±19.46[b]	98.81±18.68[b]	57.84±1.00[b]	64.26±0.03[b]	2355.62±46.69[a]
Furanic compounds					
Furan, 2-ethyl	13.63±0.48[a]	41.62±12.71[a]	20.02±2.09[a]	37.01±8.01[a]	27.33±0.74[a]
2-n-Buthyl furan	6.37±2.68[b]	16.55±3.54[a]	7.19±0.20[b]	5.66±1.25[b]	8.67±0.08[b]
Furan, 2-pentyl	802.27±302.31[a]	461.91±1.64[a]	315.37±7.84[a]	352.87±5.05[a]	435.61±73.73[a]
Furan, 2-(1-pentenyl)-(E)	27.85±5.85[a]	20.50±1.31[a]	ND	5.76±0.46[b]	21.23±2.85[a]
Total	848.05±218.07[a]	540.58±11.26[a]	342.59±7.17[a]	401.29±1.53[a]	483.28±61.79[a]
Other					
Heptane	27.06±4.62[a]	8.97±3.91[b]	6.48±0.23[b]	28.38±7.42 [a]	27.82±0.96 [a]
Octane	41.95±27.62[a]	7.67±1.31[a]	10.94±3.43[a]	17.22±3.26[a]	26.71±1.17[a]
Total	69.01±22.80[a]	16.64±3.69[a]	17.42±2.26[a]	45.61±2.95[a]	54.54±1.51[a]

The decrease of aldehydes, and specifically of hexanal, upon fermentation was previously reported with the use of different strains of yeast (Vong & Liu, 2017). Among the most abundant aldehydes present in unfermented sample, also benzaldehyde, with bitter almond notes, was detected (114.58±51.76 μg/g). Although no significant differences were highlighted, this compound seemed to decrease in fermented okara, as observed in Figure 2, contrary to what was observed in other soy products, in which benzaldehyde increase upon fermentation (Achouri et al., 2006; Dajanta et al., 2011).

A higher concentration of nonanal was found in the unfermented samples in comparison to the fermented ones. Nonanal is an aldehyde deriving from lipid degradation, that

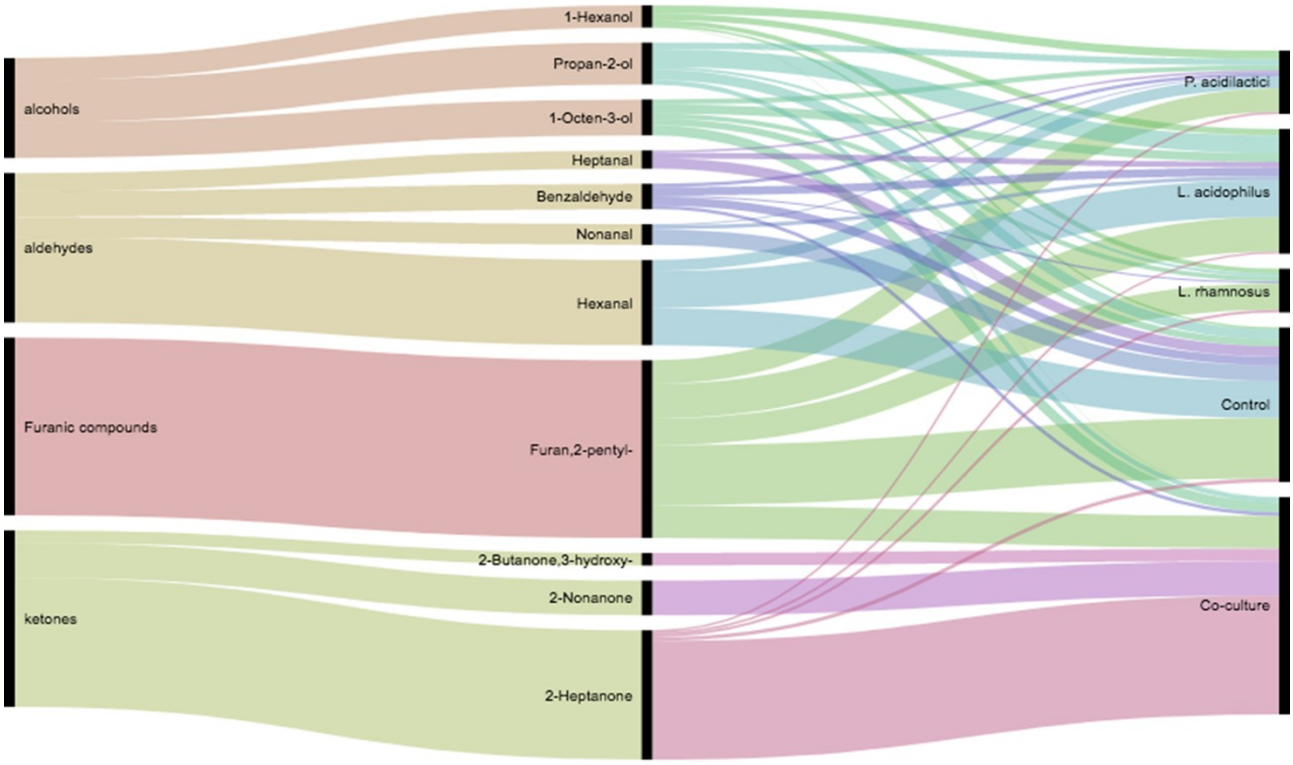
contributes to the beany aroma of legumes (Schindler et al., 2011). A significant difference was observed between the control (208.27±117.35 μg/g) and fermented samples with co-culture and *L. rhamnosus* 1473, where nonanal was completely absent. The overall decrease of aldehydes, observed in all fermented samples, may be related to contemporary formation of alcohols, via reduction mechanisms during fermentation, as shown in Table 1.

Alcohols were the second major class in the volatile fraction of okara and the most abundant compound is 1-octen-3-ol (green and mushroom notes); its formation in soy has been attributed to enzymatic reactions in soaked soybeans, a pre-treatment for soy milk manufacture (Vong & Liu, 2017). Although no significant differences among the samples were observed, SSF process with *P. acidilactici* and *L. rhamnosus* induced a decrease of about 50% of 1-octen-3-ol concentration. It is possible to hypothesize that the lower concentration of 1-octen-3-ol is associated with a lower enzymatic activity of the two species.

One of the main components of the aroma of soybean is 1-hexanol (Lee & Shibamoto, 2000). A statistically significant increase of this volatile was observed in samples fermented with *P. acidilactici* (217.53±10.72 μg/g), *L. acidophilus* (206.74±46.22 μg/g), and *L. rhamnosus* (216.96±25.33 μg/g), while an opposite behavior was observed in okara fermented with co-culture (16.01±3.58 μg/g). Stress conditions cause different cellular responses, depending on the strain which may translate into the formation of secondary metabolites, such as aromatic compounds (Filannino et al., 2014). As the synthetic mechanisms for alcohol and other volatile compounds are strain-specific, it is possible to hypothesize that, when strains are present as monoculture there is a reduction of unstable aldehydes and ketones to primary and secondary alcohols, while the synergic interaction between the strains in the co-culture may instead lead to a production of higher levels of ketones, that could be correlated to the oxidation of alcohols.

After 72 hours of incubation, a significant increase of ketones (Figure 2) was recorded in the sample fermented with co-culture, mainly ascribed to 2-nonanone and 2-heptanone (461.54±9.53 μg/g and 1581.64±61.19 μg/g, respectively).

Figure 2. Alluvial diagram showing the most representative volatile compounds for each class in fermented and unfermented (control) okara



This increase could be related to the combined metabolic activity of the strains that leads to the degradation and metabolization of the substrates, thus increasing the concentration of volatile compounds. Ketones flavor notes are generally described as desirable, and associated with sweet, fruity and creamy sensations (Y. Zhu et al., 2019). In particular, 2-butanone-3-hydroxy (acetoin), detected in samples fermented with co-culture and characterized by fatty butter taste, is widely used as flavor and fragrance in the food industry (Jia et al., 2017).

Our results were in agreement with previous studies where an increase in ketones concentration was observed after fermentation of soy-based products with *Bacillus* (Vong & Liu, 2017) and yeast (Dajanta et al., 2011).

Solid state fermentation did not significantly affect the total concentration of furan compounds, which are present at high concentrations in the control sample although a general decreasing trend was observed in all fermented samples. This class is mainly represented by furan 2-pentyl, a product deriving from the oxidation of unsaturated fatty acids, often used as a food additive due to its caramel notes. For this component, a decrease in concentration in all the fermented samples was observed.

Finally, the presence of two hydrocarbons was also observed, with no significant differences among the analyzed samples.

Phytochemical profile and LAB-derived metabolites

The fermentation effect on non-volatile organic acids and polyphenolic compounds using different LAB strains and co-culture towards okara-derived phytochemicals was evaluated through UHPLC-MS^n. At least 45 different compounds were identified, even some components specifically occurred in certain samples as a consequence of metabolic biotransformation by LAB strains (Table 2).

Table 2. UHPLC-MSn characteristics of compounds detected with their relative occurrence in unfermented and fermented okara

Compound	RT	[M-H]- (m/z)	MS^2 ions (m/z)	MS^3 ions (m/z)
Succinic acid	1.57	117	73, 99	
2-Hydroxyvaleric acid or 2-Hydroxyisovaleric acid	3.94	117	71	
Hydroxycaproic acid isomer	5.50	131	85	
2-Hydroxyisocaproic acid (Leucic acid)	5.60	131	**85**, 87, 113, 59	69
Malic acid	0.99	133	115	71
2-Hydroxy-4-(methylthio)butyric acid	3.94	149	101, 103, 107	
p-Coumaric acid	6.00	163	119	
Phenylalanine	2.87	164		
Phenyllactic acid	6.22	165	147, 119	
Tyrosine	1.48	180	**163**, 119, 136	119
***p*-Hydroxyphenyllactic acid**	4.16	181	163, 135, 113	
Azelaic acid	7.11	187	**125**, 169, 97	97, 105, 83
Citric acid	1.32	191	111, 173, 129, 87	
Tryptophan	4.10	203	159, 116, 142, 173, 129	
Indole-3-lactic acid	6.54	204	**186**, 158, 142, 160, 116	142, 158, 116, 130
Pantothenic acid	3.26	218	**88**, 146	59
Daidzein	7.85	253	209, 197, 224, 225, 226, 169, 182, 195, 145	
Genistein	9.10	269	**225**, 224, 201, 241, 181, 197, 199, 213, 169, 133, 159, 107	181, 182, 186, 195, 197, 198
Naringenin	9.00	271	151, 177, 125, 107, 165	
Glycitein	8.10	283	268	240
Kaempferol	9.18	285	241, 239, 189, 257	
Vanillic acid-*O*-hexoside	3.42	329	**167**, 123, 209	152, 123, 108
Syringic acid-*O*-hexoside	3.80	359	**197**, 182	182, 153, 138

Daidzein-*O*-hexoside	5.57	415	**253**, 295	209, 225, 180, 212, 208, 207, 196
Genistein-*O*-hexoside	6.37	431	**269**, 268, 311	224, 201, 241, 225, 240, 226, 213, 180, 169, 157, 133
Naringenin-*O*-hexoside I	5.89	433	271	151, 177
Naringenin-*O*-hexoside II	6.78	433	271	151, 177
Naringenin-O-hexoside III	7.30	433	**271**, 313	151, 177
Glycitein-*O*-hexoside	5.70	445	283	268
Kaempferol-*O*-hexoside	6.60	447	**285**, 327, 363, 256, 241	241, 257, 213, 167, 151, 256
Dihydrokaempferol-*O*-hexoside	5.25	449	**287**, 269, 259	259, 243, 269
Daidzein-*O*-acetylhexoside I	6.20	457	397, 253	
Daidzein-O-acetylhexoside II	6.30	457	253	
Daidzein-*O*-acetylhexoside III	6.40	457	253	
Daidzein-*O*-acetylhexoside IV	6.94	457	**253**, 252, 295, 397	224, 225, 197, 209, 208, 135
Genistein-*O*-acetylhexoside I	7.03	473	269	
Genistein-*O*-acetylhexoside II	7.12	473	**269**, 413	225, 240, 227, 181
Genistein-*O*-acetylhexoside III	7.83	473	**269**, 268, 311	227, 225, 224, 251, 250, 241, 133
Glycitein-*O*-acetylhexoside	6.41	487	283	268
Naringenin-*O*-acetylhexoside	7.35	475	271	151, 177, 107
Pinoresinol-*O*-hexoside	6.37	519	**357**, 475	151, 136, 327, 295, 311
Kaempferol-*O*-dihexoside	5.74	609	**285**, 429	257, 213, 151, 229, 241, 197, 200
Daidzein-*O*-hexoside derivative	4.35	623	415	253, 295
Genistein-*O*-hexoside derivative	4.78	639	**431**, 593	268, 269

The first identification step allowed the subsequent quantification of the most abundant isoflavones and some LAB-derived metabolites to unravel the putative role of LAB in the production of bioactives upon okara fermentation (Table 3).

Table 3. Concentration (◎g/g) of isoflavones and main LAB-derived metabolites recorded in unfermented (control) and fermented okara with different strains after 72 hours.

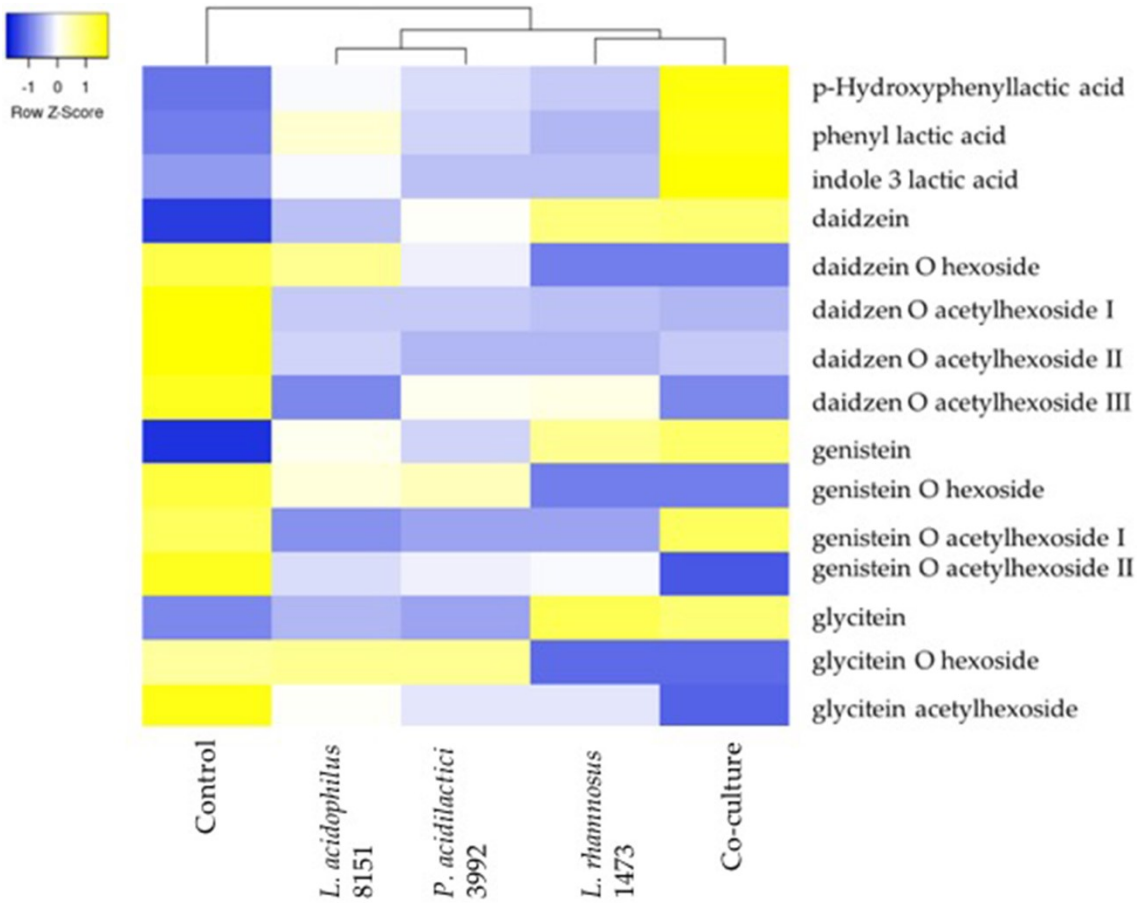
	Okara sample				
Compound	**Control**	***L. acidophilus* 8151**	***L. rhamnosus* 1473**	***P. acidilactici* 3992**	**Co-culture**
p-Hydroxy-phenyllactic acid	ND	48.54 ±3.58^{b}	30.82 ±1.93^{c}	37.98±4.17^{c}	133.95±4.42^{a}
Phenyllactic acid	ND	96.24±9.10^{b}	32.58±2.40^{c}	48.83±2.81^{c}	191.79±15.05^{a}
Indole-3-lactic acid	9.99±1.53^{d}	30.65±1.60^{b}	18.65±0.57^{c}	19.77±1.45^{c}	84.91±4.89^{a}
Daidzein	109.67±5.76^{d}	277.41±25.50^{c}	500.77±20.47^{a}	358.62±5.52^{b}	520.49±27.12^{a}
Daidzein-*O*-hexoside	33.24±2.79^{a}	26.30±2.24^{b}	ND	13.36±2.01^{c}	ND
Daidzein-*O*-acetylhexoside I	7.53±1.12^{a}	2.12±0.33^{b}	1.92±0.27^{b}	2.10±0.54^{b}	1.63±0.35^{b}
Daidzein-*O*-acetylhexoside II	6.22±0.75^{a}	1.74±0.30^{b}	1.40±0.17^{b}	1.42±0.27^{b}	1.62±0.35^{b}
Daidzein-*O*-acetylhexoside III	136.76±9.76^{a}	1.94±0.58^{c}	59.55±3.45^{b}	56.40±4.76^{b}	3.76±1.24^{c}

Genistein	108.01±2.80^{e}	376.37±22.62^{c}	494.79±15.67^{b}	303.28±7.72^{d}	532.60±16.61^{a}
Genistein-*O*-hexoside	25.64±1.20^{a}	13.45±0.49^{c}	ND	16.09±2.20^{b}	ND
Genistein-*O*-acetylhexoside I	11.11±0.14^{a}	4.20±0.44^{b}	4.77±0.52^{b}	4.83±0.42^{b}	11.10±0.78^{a}
Genistein-*O*-acetylhexoside II	152.17±1.56^{a}	59.87±6.63^{c}	71.64±3.78^{b}	68.98±5.91^{b,c}	11.03±0.84^{e}
Glycitein	0.52±0.07^{d}	1.55±0.32^{c}	6.48±0.41^{a,b}	1.22±0.16^{c,d}	5.85±0.60^{a}
Glycitein-*O*-hexoside	6.60±0.24^{a}	6.97±0.74^{a}	ND	6.76±0.32^{a}	ND
Glycitein-*O*-acetylhexoside	12.61±0.26^{a}	6.03±0.53^{b}	5.04±0.26^{c}	5.02±0.46^{c}	0.85±0.04^{d}

Mean values ± SD, n=3 for control and n= 4 for fermented samples. Different letters indicate significantly different values (p<0.05); ND: not detected

Unfermented okara contained several polyphenols, most notably isoflavones, which mainly occurred as *O*-glycosides. Isoflavone-*O*-glycosides were converted by LAB β-glucosidases in their aglycone forms. In all fermented samples, a decrease of glycosides (daidzein-*O*-hexoside; daidzein-*O*-acetylhexoside I, II, III; genistein-*O*-hexoside; genistein-*O*-acetylhexoside I, II; glycitein-*O*-hexoside; glycitein-*O*-acetylhexoside) and the consequent increase of each respective aglycone (daidzein; genistein; glycitein) was observed, in comparison to the control (Figure 3).

Figure 3. Heatmap visualization of the phytochemical compounds of fermented and unfermented (control) okara, based on the Euclidean distance. The color scale represents the scaled abundance of each variable, with yellow color indicating high abundance and blue color indicating low abundance.



The deglycosylation of isoflavones was previously observed in okara and soy products after fermentation with different monoculture of yeast and LAB (Li et al., 2020; Queiroz Santos et al., 2018), but, to the best of our knowledge, the current study reveals the high potential of LAB co-culture to convert isoflavones for the first time.

Notably, daidzein-*O*-glycosides were mainly converted into free daidzein in all fermented samples with significant differences between LAB strains, reaching the highest concentration after fermentation with *L. rhamnosus* (500.77±20.47 μg/g) and co-culture (520.49±27.12 μg/g). A similar trend was observed for genistein, although fermented samples with co-culture strains showed a significant higher concentration (532.60±16.61 μg/g) of this isoflavone with respect to samples biotransformed by *L. rhamnosu*s (494.79±15.67 μg/g). Genistein and daidzein were by far the most abundant isoflavones upon fermentation, while glycitein was barely recovered, contributing to ~ 1% of the overall isoflavone aglycones upon SSF. Although this is the first evidence on bacteria, a synergistic

effect of co-culture corresponding to a high bioconversion of isoflavones has already been reported in okara fermented with fungi (Vong et al., 2018).

The capability of both *L. rhamnosus* and co-culture to produce higher levels of aglycones could represent a basis to investigate okara as a functional ingredient, given the putative better absorption of aglyconic isoflavones in the upper gastrointestinal (GI) tract with respect to the corresponding glycosides, even if some literature works didn't reach the same conclusion (Izumi et al., 2000; Koh & Mitchell, 2007; Okabe et al., 2011; Rüfer et al., 2008). Results of these studies are difficult to compare as different delivery forms of isoflavones, such as pure compounds, tablets or soy-based products, were investigated. However, focusing only on soy-based products, several human feeding studies highlighted the improvement of isoflavone bioavailability in the first GI tract upon soy fermentation as a result of the higher aglycone content than the unfermented counterparts (Koh & Mitchell, 2007; Okabe et al., 2011).

Although isoflavones were the main polyphenols in the okara samples investigated in the present study, single and co-culture LAB strains similarly interacted with other minor flavonoids and phenolic acids (Table S2). The flavanone naringenin was significantly higher (as chromatographic area) in both *L. rhamnosus* and co-culture okara samples with respect to the other fermentations, whereas all three *O*-glycosylated isomers of naringenin dropped upon SSF, two of these reaching non-detectable levels in the okara fermented with co-culture strains. Besides glycosylated flavonoids, unfermented okara contained phenolic acids such as vanillic and syringic acids, both in *O*-glycosidic form, which significantly decreased after SSF only in *L. rhamnosus* and co-culture strains (Table S2).

Besides the increase of aglycone isoflavone and naringenin, released through LAB-mediated deglycosylation, the SSF of okara led to the formation of LAB-derived smaller phenolic metabolites such as indol-3-lactic, phenyllactic and *p*-hydroxyphenyllactic acids, which were almost completely absent in the unfermented samples. The capacity of LAB to produce phenyllactic acid during fermentation had been previously reported, in particular for *L. rhamnosus* 1473 (Ricci et al., 2019), and in the current study, these compounds reached the highest concentration after co-culture fermentation, i.e. 191.79±15.05 for phenyllactic acid

and 133.95±4.42 μg/g for *p*-hydroxyphenyllactic acid. Their production may be ascribed to the metabolism of amino acids by LAB. In particular, the former is produced from the metabolism of phenylalanine, while the latter from tyrosine metabolism. The recovery of phenyllactic acid in fermented okara could be interesting, since previous studies have shown that this phenolic acid has antimicrobial activity against both Gram-positive and Gram-negative bacteria, and inhibitory activity against a wide range of fungi, isolated from baked goods, flours and cereals, including some mycotoxigenic species. Many strains of the *Lacticaseibacillus* genus can produce phenyllactic and *p*-hydroxyphenyllactic acids, which contribute to preserving the quality of food, maintaining the sensorial characteristics typical of fermented products (Sangmanee & Hongpattarakere, 2014; Valerio et al., 2004). The presence of these compounds could be useful in the case of okara, which presents itself as an easily perishable and microbiologically unstable material.

The SSF led to a further increase of another LAB-derived metabolite, namely indole-3-lactic acid, produced *via* tryptophan catabolism (Roager & Licht, 2018). This catabolite can be produced by yeasts and bacterial species and can inhibit the growth of Gram-positive and Gram-negative bacteria (Naz et al., 2013), as well as by acting as an antifungal compound against *Penicillium* strains (Honoré et al., 2016). Indole-3-lactic acid reached the highest concentration in the okara fermented with co-culture (84.91±4.89 μg/g), displaying significant differences when compared to single LAB strains and control (Table 3). Accordingly, an opposite trend was observed for the precursor tryptophan, which showed a significant prominent drop (as chromatographic peak area) in the co-culture fermented samples (Table S2). Several promising studies have highlighted the putative bioactivity of indole-3-lactic acid through *in vitro* and *in vivo* experiments (Roager & Licht, 2018). Some *in vitro* experiments showed that indole-3-lactic acid can reduce inflammation (Cervantes-Barragan et al., 2017; Wilck et al., 2017) and this behavior was partially confirmed in human studies. Moreover, circulating indole-3-lactic acid was significantly lower in plasma of obese subjects than in non-obese ones, and was paralleled by lower serum levels of inflammatory markers (Cussotto et al., 2020). The putative anti-inflammatory activity elicited by indole-3-lactic acid was further supported in an intervention study, as its plasma levels significantly

increased in humans that followed a Mediterranean diet of four days when compared to a control fast-food diet in a crossover design (C. Zhu et al., 2020).

Another amino acid-derived metabolite, specifically from leucine, namely 2-hydroxyisocaproic acid (leucic acid), was undetectable in the control sample while it was recovered in all fermented okara samples, even if differences emerged between LAB strains. In detail, okara fermented with *L. acidophilus* showed the highest recovery of this α-hydroxy acid after SSF with co-culture (Table S2), in agreement with previous works on leucic acid production by different LAB strains (Axel et al., 2016; Koistinen et al., 2018). The ability to biotransform compounds present in substrates even in absence of replication, like that occurred with *L. acidophilus*, was recently reported (Ricci, Levante, et al., 2018).

Conclusions

The present study explored the use of LAB to ferment okara: the metabolic activity of LAB resulted in fermented final products with different chemical compositions and biological activity. Although the bioprocess occurred especially at a high replication rate, also in the case of non-multiplying bacterial cells an increase of specific metabolites was observed.

Exploring different strains and their combinations, the co-culture containing *L. acidophilus*, *L. rhamnosus* and *P. acidilactici* was the best starter candidate due to its ability to significantly modify the aromatic and polyphenolic profile of raw material. Besides the optimal growth performance, a decrease of off-flavor (hexanal, nonanal) and a large conversion of isoflavones in their aglycone forms were obtained. Moreover, a notable production of LAB-derived metabolites such as indol-3-lactic, phenyllactic and *p*-hydroxyphenyllactic acids, that can exert a human biological activity or antimicrobial activity, was observed.

Based on the obtained results, solid state fermentation may represent an innovative strategy for the reuse of okara with the final goal of the recovery of possible functional ingredients.

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Supplementary materials

Table S1 Assignment of GC-MS signals

Peak no.	Identification	LRI	Identification Method	Reference
1	Heptane	790	MS	
2	Octane	829	MS	
3	Acetone	843	MS + LRI	Castello et al., 1991
4	2-Butanone	911	MS + LRI	Bianchi et al., 2007
5	2-Methyl propanal	913	MS + LRI	Bianchi et al., 2007
6	Propan-2-ol	939	MS + LRI	Castello et al., 1991
7	Ethanol	955	MS + LRI	Castello et al., 1991
8	Furan, 2-ethyl	976	MS + LRI	Girard & Durance, 2000
9	Pentanal	1012	MS + LRI	Rochat & Chaintreau, 2005
10	Hexanal	1107	MS + LRI	Rochat & Chaintreau, 2005
11	2-n-Buthyl furan	1146	MS + LRI	Elmore et al., 2005
12	2-Heptanone	1210	MS + LRI	Rochat & Chaintreau, 2005
13	Heptanal	1213	MS+ LRI	Rochat & Chaintreau, 2005
14	1-Butanol,3-methyl	1231	MS + LRI	Bianchi et al., 2007
15	1-Pentanol	1232	MS + LRI	Girard & Durance, 2000
16	Furan, 2-pentyl	1255	MS + LRI	Girard & Durance, 2000
17	2-Octanone	1310	MS + LRI	Rochat & Chaintreau, 2005
18	Octanal	1313	MS + LRI	Rochat & Chaintreau, 2005
19	2-Butanone,3-hydroxy-	1315	MS + LRI	Bianchi et al., 2007
20	Furan, 2-(1-pentenyl)-(E)	1325	MS	
21	2-Heptenal	1351	MS + LRI	Rochat & Chaintreau, 2005
22	1-Hexanol	1375	MS + LRI	Girard & Durance, 2000
23	3-Octanol	1415	MS + LRI	Chung et al., 2011
24	Nonanal	1419	MS + LRI	Rochat & Chaintreau, 2005
25	3-Octen-2-one	1434	MS + LRI	Elmore et al., 2005
26	2-Octenal (E)	1458	MS + LRI	Rochat & Chaintreau, 2005
27	1-Octen-3-ol	1473	MS	
28	1-Heptanol	1478	MS + LRI	Bianchi et al., 2007
29	Furfural	1495	MS + LRI	Bianchi et al., 2007
30	4-Ethylciclohexanol	1506	MS	
31	Decanal	1526	MS + LRI	Rochat & Chaintreau, 2005
32	Benzaldehyde	1556	MS + LRI	Da Porto et al., 2003
33	1-Octanol	1581	MS + LRI	
34	3,5-Octadien-2-one	1601	MS	
35	2-Undecanone	1626	MS + LRI	Rochat & Chaintreau, 2005
36	2-Octen-1-ol	1640	MS + LRI	Wong & Lai, 1996
37	Dodecanal	1739	MS + LRI	Rochat & Chaintreau, 2005
38	Benzaldehyde, 4-ethyl	1742	MS	
39	Benzaldehyde, 2,5-dimethyl	1742	MS	
40	2,4-Decadienal	1797	MS + LRI	Rochat & Chaintreau, 2005
41	Benzyl Alcohol	1909	MS + LRI	Loughrin et al., 1990
42	Phenylethyl Alcohol	1945	MS + LRI	Loughrin et al., 1990

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Table S2. Chromatographic area of minor components in unfermented and fermented okara samples analyzed through UHPLC MSn

Compound	Okara sample				
	Control	*L. acidophilus* 8151	*P. acidilactici* 3992	*L. rhamnosus* 1473	Co-culture
Tryptophan	19960±415^{a}	11295±2053^{c}	14718±1138^{b}	17904±1670^{a,d}	2668±678^{e}
Naringenin	12719±107^{d}	30501±2479^{b}	20044±3301^{c}	52930±2500^{a}	51338±5024^{a}
Naringenin-*O*-hexoside I	13162±440^{a}	8412±660^{c}	11799±1085^{b}	2098±225^{d}	ND
Naringenin-*O*-hexoside II	15803±20^{a}	4353±796^{c}	6535±784^{b}	ND	ND
Naringenin-*O*-hexoside III	4496±591^{a}	5084±643^{a}	4950±932^{a}	3702±258^{b}	3267±170^{b}
2-Hydroxyvaleric acid or 2-Hydroxyisovaleric acid	ND	4984±656^{b}	ND	NQ	6351±531^{a}
2-Hydroxyisocaproic acid (Leucic acid)	ND	38481±2727^{a}	4979±760 ^{c,d}	7986±1727^{c}	32467±2063^{b}
Vanillic acid-*O*-hexoside	7942±245^{a}	8832±1414^{a,b}	7140±393^{a,c}	ND	ND
Syringic acid-*O*-hexoside	13780±273^{a}	13349±1364^{a}	12947±510^{a}	ND	2364±472^{b}

Data are expressed as arbitrary units (mean values ± sd, with n=3 for control and n= 4 for fermented okara samples). nd: not detected; nq: s/n too low (<10). different letters showed significant differences by ANOVA at $p < 0.05$.

Chapter 2.3

Exploring the potential of lactic acid fermentation for the recovery of exhausted vanilla beans

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Manuscript in preparation

Abstract

The market value of vanilla is constantly growing, as it is the aroma most appreciated by consumers worldwide. The key component of the aroma of vanilla beans is vanillin, which can be directly extracted from the plant, produced by chemical synthesis, or by bioconversion of natural precursors. Due to the increasing consumers' demand for products labeled as "natural", extraction from vanilla pods results in a more valuable aroma source. Once the extraction is completed, what remains are the exhausted beans that still contain small seeds and other compounds, including varying amounts of vanillin trapped in the cellular structures of the plant.

The application of fermentation of exhausted vanilla beans is proposed here as a strategy to recover "natural" vanillin and other valuable aroma compounds as a result of metabolic conversion by microorganisms.

In particular, a Design of Experiment (DoE) approach was used to screen a library of Lactic Acid Bacteria (LAB) strains to identify the best condition of fermentation by modification of different parameters.

A comparison between mono and co-culture was assessed. Moreover, a sensory panel test and the evaluation of the aromatic components by Solid Phase Micro Extraction-Gas Chromatography-Mass Spectrometry analysis were carried out to better understand the modification of the aroma profile after fermentation. Fermentation with LAB changed the volatile profile and sensory characteristics of the exhausted vanilla beans and represents a promising method for the valorization of these by-products.

Introduction

Vanilla (*Vanilla planifolia*) is a tropical orchid famous worldwide for its aroma. The market value of vanilla is constantly growing as it is the most appreciated aroma by consumers and therefore used in the food field for desserts, ice creams, and for soft drinks and spirits (Baqueiro-Peña & Guerrero-Beltrán, 2017). In 2017, the global vanilla market size was 65 million US$ but it is expected to grow in the next years reaching 100 million US$ in 2025 (Report, 2020).

Among the main components of the aroma, the most important and abundant in vanilla beans is vanillin. Vanillin can be directly extracted from the plant, can be produced by chemical synthesis, or by bioconversion of ferulic acid (Brunati et al., 2004; Karmakar et al., 2000; Lesage-Meessen et al., 1996). To meet the growing global demand, chemical synthesis of vanillin has become the major source, using guaiacol and lignin as starting materials. This choice has disadvantages due to the large quantities of pollutants, which make it an unsustainable environmental technique (Gallage & Møller, 2017). Moreover, in recent years consumers have become more aware of products labeled as 'natural', thus favoring those products using aroma extracted directly from the vanilla beans. Once the beans have been harvested, they must ripen and begin the curing process that consists of different steps in which vanilla beans develop their typical aroma due to enzymatic processes mainly linked to the action of β-glucosidases presents in the vegetal tissue (Frenkel et al., 2011). The extraction consists in leaving the pods in a solution of water and ethanol, but vacuum can be applied to enhance the process (Ramachandra Rao & Ravishankar, 2000). Once the extraction is complete, what remains are the exhausted beans that still contain small seeds. The small vanilla seeds are obtained from the beans just after their extraction with a complex separation process and are used by the food industry to accomplish consumers' demand for increased naturalness of foods. At the end of extraction process, other compounds, and varying amounts of vanillin, remain trapped in the cellular structures of the plant (Pardío et al., 2018). Another technique that can be used to produce "natural" vanillin is fermentation. This technique allows the conversion of natural substances such as lignin, ferulic acid, eugenol present in the beans of vanilla thanks to the enzyme portfolio of

microorganisms (Kaur & Chakraborty, 2013). Recent studies reported the conversion into vanillin by microorganisms using agro-industrial waste like cereal bran, sugar beet pulp, or rice brain oil (Chattopadhyay et al., 2018; Zamzuri & Abd-Aziz, 2013).

In recent years, solid-state fermentation has been widely used for the recovery of bioactive molecules from agro-industrial by-products using lactic acid bacteria (LAB) (Hadj Saadoun, Calani, et al., 2021; Hadj Saadoun, Ricci, et al., 2021; Ricci, Cirlini, et al., 2019; Ricci, Diaz, et al., 2019). LAB consists of a heterogeneous group of Gram-positive bacteria recognized as GRAS, widely used in the food industry as starters as they can grow on many different carbon sources and have a good tolerance to environmental stresses (Mazzoli et al., 2014). During the fermentation process, many bioactive compounds are produced by LAB such as antioxidants, bacteriocins but also volatile compounds (Filannino et al., 2014; Ricci et al., 2020).

For these reasons, fermentation of exhausted vanilla beans could be a valuable strategy for the recovery of valuable aroma compounds which are not primarily extracted. For the fermentation study, two different byproducts were selected: one consisted of the whole dried and grinded exhausted vanilla beans with seeds, and the other one without seeds, to determine if their presence in the fermented substrate enhances the overall flavor performance. To the best of our knowledge, no studies are present in literature regarding the fermentation of vanilla by-products. To identify the best fermentation condition and to screen strains of LAB present in culture collection, we evaluated different parameters using the Design of Experiments (DoE).

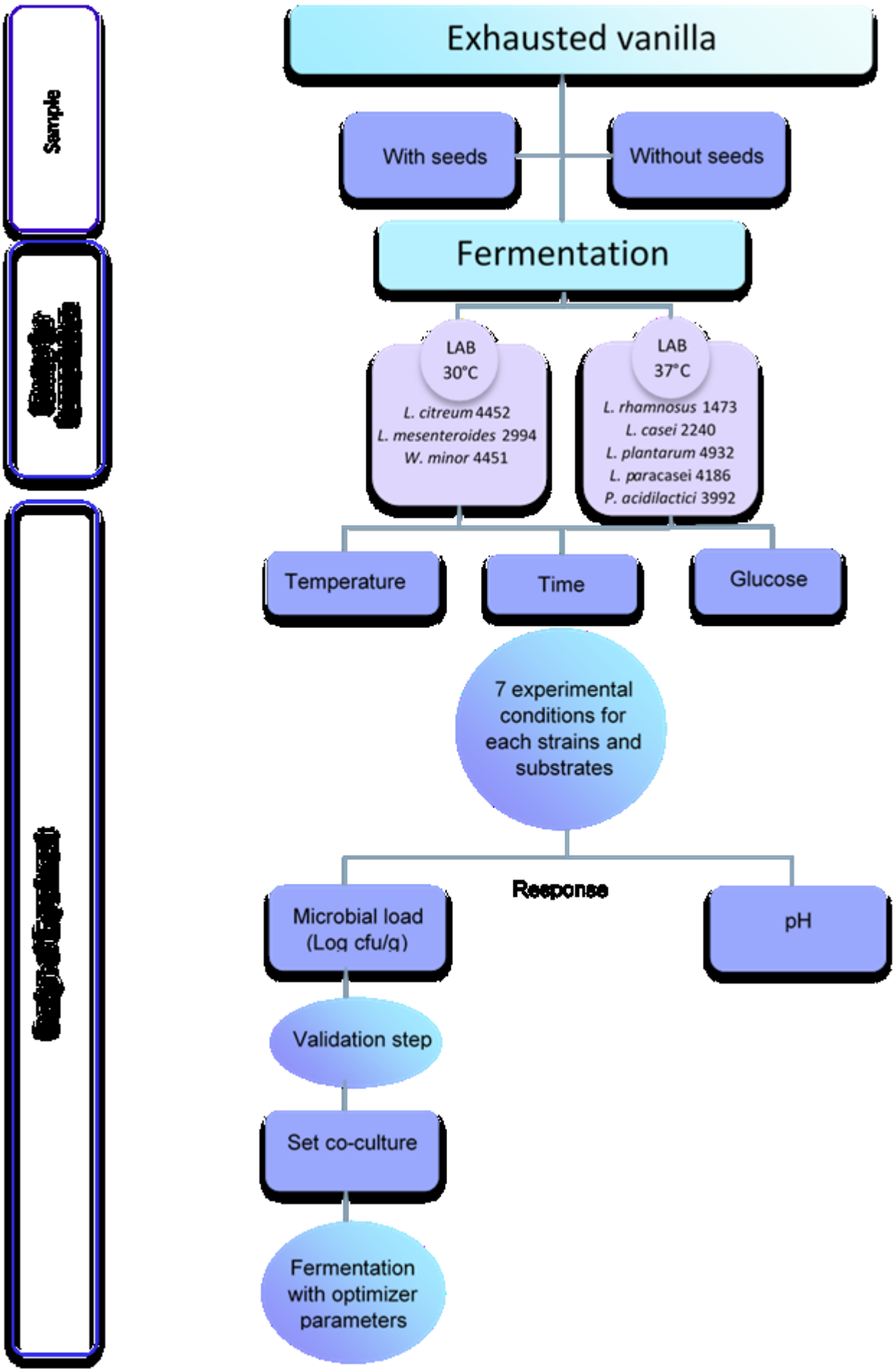
The fermentation of the substrates was monitored by evaluating the modification of pH and microbial growth. Once optimal conditions were identified for each strain, we evaluated the co-culture growth of the strains previously studied as mono-culture. Moreover, a sensory panel test and the evaluation of the aromatic components by Solid Phase Micro Extraction-Gas Chromatography-Mass Spectrometry (SPME-GC/MS) analysis were carried out to better understand the modification of the vanilla beans after fermentation.

Materials and Methods

Substrates

The substrate under study was exhausted vanilla beans after the vanillin extraction. Figure 1 shows the experimental procedure. Two different samples were taken into consideration: vanilla beans with seeds (S) and without seeds (NS). Both substrates were autoclaved before fermentation with the selected strains. Samples were provided by Giotti- McCormick company.

Figure 1. Experimental procedure for fermentation of exhausted vanilla beans


Sample
Exhausted vanilla
With seeds
Without seeds
Fermentation
LAB
30°C
L. citreum 4452
L. mesenteroides 2994
W. minor 4451
LAB
37°C
L. rhamnosus 1473
L. casei 2240
L. plantarum 4932
L. paracasei 4186
P. acidilactici 3992
Temperature
Time
Glucose
7 experimental conditions for each strains and substrates
Response
Microbial load (Log cfu/g)
pH
Validation step
Set co-culture
Fermentation with optimizer parameters

Bacterial strains

Eight bacterial strains isolated from different niches and belonging to different LAB species were used for fermentation (Table 1).

Table 1. List of lactic acid bacteria strains used for fermentation of exhausted vanilla beans

Strains	Isolated from	Optimal growth temperature
***Leuconostoc citreum* 4452**	Sour dough	30°C
***Leuconostoc mesenteroides* 2194**	Grana Padano cheese	30°C
***Weissella minor* 4451**	Sour dough	30°C
***Lacticaseibacillus rhamnosus* 1473**	Parmigiano Reggiano cheese	37°C
***Lacticaseibacillus paracasei* 4186**	Pecorino Toscano cheese	37°C
***Lacticaseibacillus casei* 2240**	Parmigiano Reggiano cheese	37°C
***Lactiplantibacillus plantarum* 4932**	Minas cheese	37°C
***Pediococcus acidilactici* 3992**	Grana Padano cheese	37°C

All the strains belong to the University of Parma Culture Collection (UPCC) of the Department of Food and Drug. The bacterial stock cultures were maintained as frozen at −80 ◦C in De Man Rogosa and Sharpe (MRS) broth (Oxoid, Basingstoke, UK) added with 12.5% glycerol (v/v).

Before fermentation, the strain was transferred twice in MRS broth (3% v/v) and incubated for 24 h at the optimal temperature. Afterward, 200 µl of culture broth was inoculated in 6 mL of MRS broth and incubated at 30-37°C for 15 h to obtain a cell concentration of 9 Log CFU/mL. Bacterial culture was centrifuged (10,000rpm, 10min, 4°C), washed twice in Ringer solution (VWR, UK), and suspended in sterile bidistilled water. Before the inoculum in the samples, substrates were sterilized in autoclave at 121°C for 21 min. Each culture was then inoculated into 10 g of vanilla beans to reach 7 Log cfu/g.

Experimental Design of Fermentation

Experiments were designed around a mathematical model using the software MODDE Pro v12.0.1 (MKS Umetrics, Umeå, Sweden). The model was designed around three quantitative factors: temperature, time of fermentation, and the concentration of glucose added to the substrates.

The measured responses were the microbial load (Log cfu/g) and the variation of pH. Response surface methodology (RSM) model was drawn up with a fractional factorial design. The fractional factorial design is a screening model often applied to overview a biological phenomenon, at the same time allowing the definition of optimum value (Gunst & Mason, 2009).

Each strain was used for the fermentation of the substrate(s) under the conditions defined by the DoE, following the matrix generated from the software with different temperatures of incubation, according to the optimal ones for each strain (Figure 2 and 3). The microbial load was evaluated at the end of the incubation time, through a plate count on MRS agar. Plates were incubated at 30-37°C for 48 hours.

The pH measurement was performed in the sample diluted 1:10 in distilled water and the analysis was conducted using a pH meter (Mettler Toledo). The pH was evaluated in the unfermented sample (control) and after fermentation.

Figure 2. Summary of experimental design of strains with 37°C as optimal growth temperature

Exp No	Exp Name	Run Order	Incl/Excl	Temperature	Time	Glucose
1	N1	4	Incl	32	30	5
2	N2	1	Incl	42	30	0
3	N3	3	Incl	32	120	0
4	N4	2	Incl	42	120	5
5	N5	5	Incl	37	75	2,5
6	N6	6	Incl	37	75	2,5
7	N7	7	Incl	37	75	2,5

Figure 3. Summary of experimental design of strains with 30°C as optimal growth temperature

Exp No	Exp Name	Run Order	Incl/Excl	Temperature	Time	Glucose
1	N1	1	Incl	25	30	5
2	N2	2	Incl	35	30	0
3	N3	3	Incl	25	120	0
4	N4	7	Incl	35	120	5
5	N5	5	Incl	30	75	2,5
6	N6	4	Incl	30	75	2,5
7	N7	6	Incl	30	75	2,5

Once the data were obtained for each strain, they were used to build a model, producing a response surface that gave us indications for obtaining the maximum response, i.e. the highest microbial load. According to the indications produced using the model in combination with a response optimization algorithm, growth conditions were postulated for the set-up of microbial co-cultures.

Sensory analysis

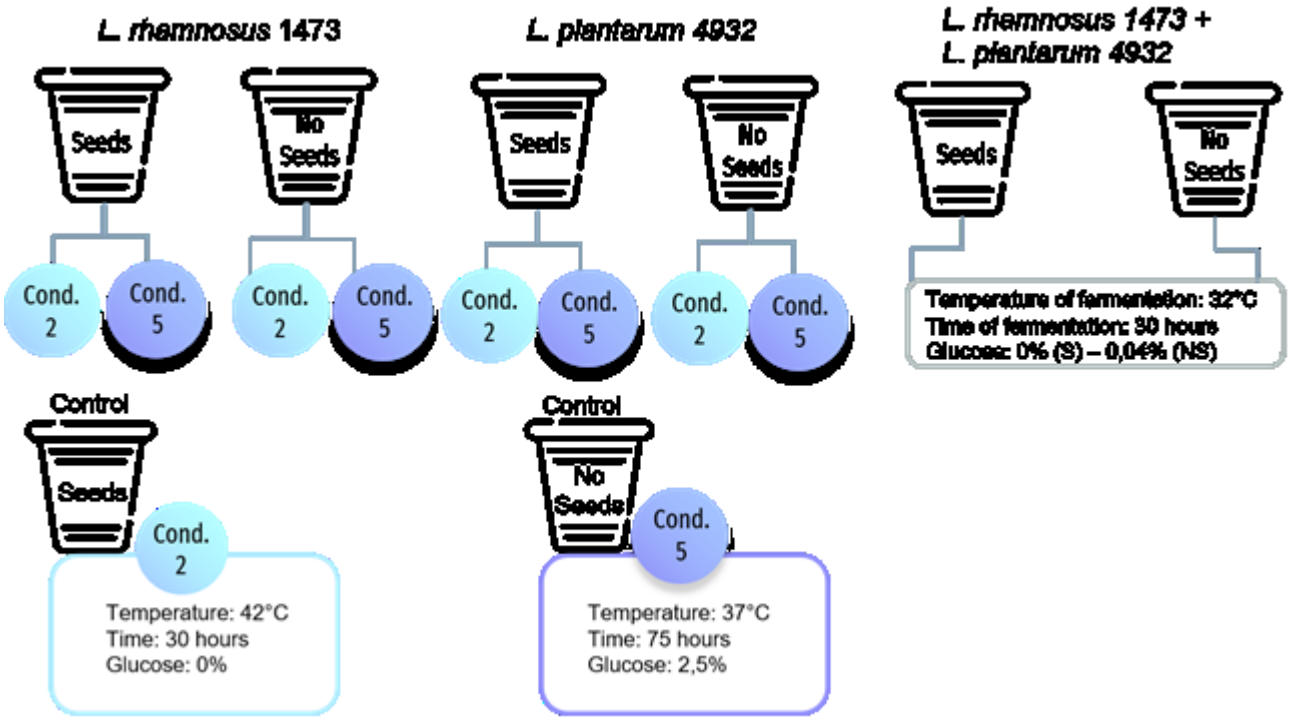
The fermented samples were evaluated through a sensory analysis to understand if the fermentation can enhance the sweet taste, for future identification of natural sweeteners deriving from this process, and to identify aromatic notes of interest that can be characterized and extracted in a subsequent study.

The samples (Figure 4) were evaluated by a panel of 12 trained sensory assessors Giotti-McCormick Company, in two replicates for their aroma (AR), flavour (FL) and aftertaste (AT). A Preference Maps test was carried out using the Sorting techniques (Greenhoff, K., & MacFie, H. J. H., 1994).

The order of sample presentation was randomized across assessors. Results were collected via the Compusense data capture system and were analyzed with XLSTAT using analysis of variance (ANOVA) ($p<0.05$).

The panelists received seven different samples of vanilla beans that differed for strains used and experimental conditions of fermentation. For this evaluation, we chose the following samples: for each monoculture, we selected condition n. 2, with no addition of glucose and fewer hours of fermentation; condition n. 5, performed at the optimal growth temperature for each strain, with the addition of sugar and longer fermentation time. For the co-culture, we selected the optimal condition generated by DoE prediction. Finally, two control samples (unfermented) were prepared for conditions n.2 and n.5.

Figure 4. Example of experimental conditions used for sensory analysis for strains with the optimal temperature of 37°C. S is the sample with seeds; NS is the sample without seeds.



Samples were tested in a solution of water and glucose (3°Bx), presented with a three-digit numerical code in sequential and balanced order for all participants. The "Compusense" Sensory Analysis software was used to carry out the test and the PCA (Principal Component Analysis).

Analysis was conducted both for vanilla beans with seeds (S) and without seeds (NS).

Characterization of the volatile fraction

Volatile compounds produced by inoculated bacteria were analyzed using a gas chromatography device coupled with a mass spectrometer. Briefly, 1 g of vanilla sample was weighed into a 20 ml vial, with 3,80 mL of distilled water, 1,60 g of NaCl, and 0,01 g of Ethyl nonanoate standard. The vial was heated at 40 °C for 50 min to equilibrate the system. The SPME fiber, 85 µm carboxen/polydimethylsiloxane Stable Flex™ (Supelco), was inserted through the septum and exposed in the headspace of the vial for 25 min, to allow absorption of the volatile compounds onto the SPME fiber. The SPME fiber was then

introduced into the injector port of the gas chromatograph for 5 min in splitless mode, set at 280 °C, to desorb the volatile compounds. The desorbed components were analyzed on a capillary column. Compounds identification was based on a comparison of retention indices (RI), mass spectra. Data were reported as ppm for each compound detected.

Results

LAB growth assessment in exhausted vanilla beans without seeds (NS) using DoE

To identify the optimal fermentation parameters, seven experimental conditions (Figure 2 and 3) were assessed for each strain, combining time and temperature of fermentation, and addition of glucose, for a total of 56 experiments. Experiments were distinguished between strains with optimum growth temperature at 37°C (Table 2) and strains with optimum at 30°C (Table 3). Experimental conditions n. 5, 6, and 7 for both tables are biological replicates to account for natural variance and represent the central point of the experimental model.

Table 2. Experimental condition for microorganisms with an optimum at 37°C. For each strain, the pH and cellular concentration reached at the end of the fermentation time are reported according to all experimental conditions.

				***L. rhamnosus* 1473**		***L. paracasei* 4186**		***L. casei* 2240**		***L. plantarum* 4932**		***P. acidilactici* 3992**	
exp N.	Tem. (°C)	time (h)	Glucose (%)	pH	Log cfu/g	pH	Log cfu/g	pH	Log cfu/g	pH	Log cfu/g	pH	Log cfu/g
1	32	30	5	6.47	7,74	6.27	7,69	6.04	8,18	5.90	7,30	6.37	7,24
2	42	30	0	4.93	7,38	4.97	7,01	4.67	8,04	4.97	7,26	4.95	5,41
3	32	120	0	6.56	7,22	5.51	7,36	5.01	7,06	5.04	6,31	5.48	6,56
4	42	120	5	6.57	6,10	6.31	3,00	5.52	4,18	6.64	4,00	6.10	4,88
5	37	75	2,5	5.01	8,22	6.66	3,00	4.90	8,33	6.77	4,00	6.37	6,24
6	37	75	2,5	5.10	8,33	6.64	3,00	4.97	8,26	6.73	4,00	6.47	6,06
7	37	75	2,5	5.14	8,29	6.60	3,00	4.88	8,24	6.80	4,00	6.53	6,33

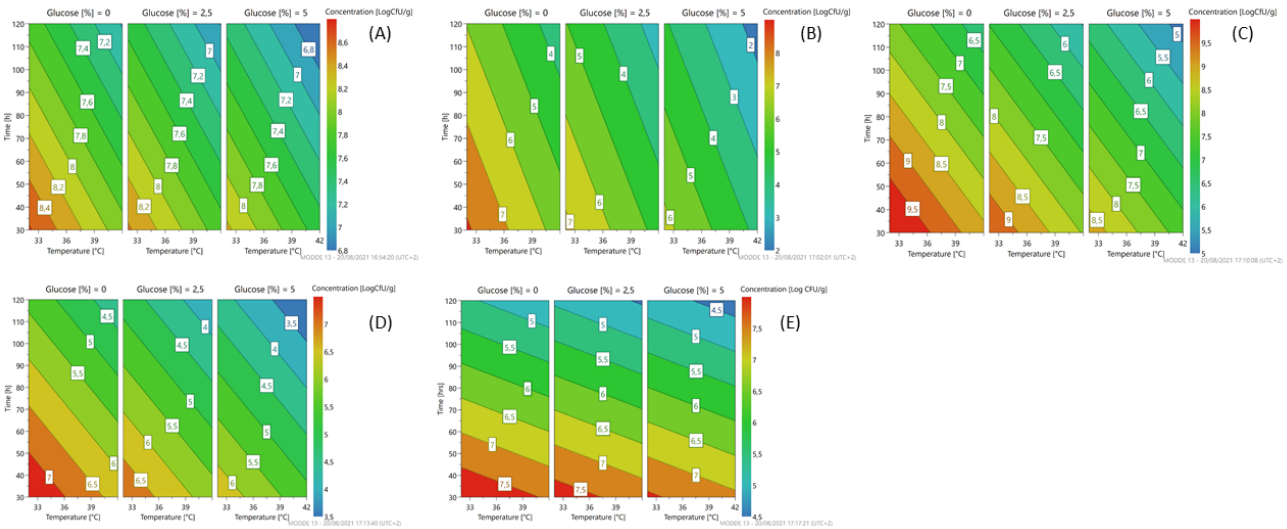
Considering the strains with optimum temperature 37°C, samples fermented with *L. rhamnosus* 1473, showed the highest microbial load in conditions 5 to 7, corresponding to the optimal growth temperature of the strains, a medium level of time of fermentation (75

h), and glucose addition. On the other hand, condition 4, where all factors were set at the maximum values, led to a decrease in microbial concentration from the original inoculum, due to cell lysis. Variations in pH are not easy to interpret: generally, microbial growth is associated with a pH decrease, but the extent of acidification can vary according to growth conditions and substrate. The control sample (seeds and without seeds) has a pH of around 6,5. In conditions n.1, 2, and 3 where a similar microbial load is observed (7 Log cfu/g), a marked pH decrease was observed only for condition n.2. For this reason, the pH response was difficult to model and predict by the software, so we focused mostly on cell concentration-response.

The Response Surface Methodology (RSM) is a valid tool as it defines the effect of the variables in combination, and it permits to describe the fermentation process for all the strains.

Looking at Figure 5A, we can see that the percentage of glucose is the factor that most regulates the change in the growth of the microorganism. At a lower glucose concentration, there is a higher growth prediction (red/orange zone). Even temperature and time of fermentation, if reduced, lead to a greater increase of cell concentration of the strain.

Figure 5. A response contour plot of concentration (Log cfu/g) of *L. rhamnosus 1473* (A); *L. paracasei 4186* (B); *L. casei 2240* (C); *L. plantarum* 4932 (D); *P. acidilactici* 3992 (E) in sample without seeds, with time and temperature as main factors and with glucose variation



This information was collected and used by the software to generate optimization conditions.

Experiments conducted with *L. paracasei* 4186, highlight different trends (Table 2). In this case, the strain shows lower adaptability to the substrates, and only in condition n.1 an increase in microbial load was observed, while in most of the other cases there was cell lysis. Also for this microorganism, the value of pH decreased in condition n.2, even if no growth was recorded.

As the response surface model shows (Figure 5B), the maximum growth is possible by setting the minimum time and temperature of fermentation and without the addition of glucose.

Fermentation with *L. casei* 2240 highlights a similar trend as for *L. rhamnosus* 1473. In this case, the strain was able to grow and replicate in almost all conditions except for the n.3 and n.4, with a long time of fermentation, where cell lysis occurs. Differently from the previous cases, here there is a different trend for pH value. In all conditions, it was recorded a lower pH value even in condition n.4. Only in the first experiment (n.1), the pH remains unchanged even if an increase of microbial load is observed. As reported in Figure 5C, we noticed as also for *L. casei* 2240 the maximum response of microbial concentration is predicted with minimum addition of glucose and less time of fermentation (red zone).

Experiments conducted with *L. plantarum* 4932 show no significant growth for all conditions. The three center points (n. 5, 6, and 7) highlight a great reproducibility of the model but also indicates that the optimal condition was not sufficient for the strain's growth. In this case, the value of pH decreased under the fermentation conditions n.1, n.2, and n.3. The response surface (Figure 5D) gives us a similar indication as to the previous, reducing the concentration of glucose, time, and temperature of fermentation gives an increase of microbial load, even if is not the most suitable species as for *L. paracasei*.

The last bacteria with an optimum temperature of 37°C was *P. acidilactici* 3992 (Figure 5E). This microorganism showed a similar trend to the previous one, with cellular lysis for almost all conditions and acidification of the substrates, especially in condition n. 2.

Moving to the strains with the optimum at 30°C we noticed a different trend, compared to the previous ones (optimum 37°C), in the measured pH values. For these strains, no decrease in pH value was recorded in any of the tested conditions (Table 3).

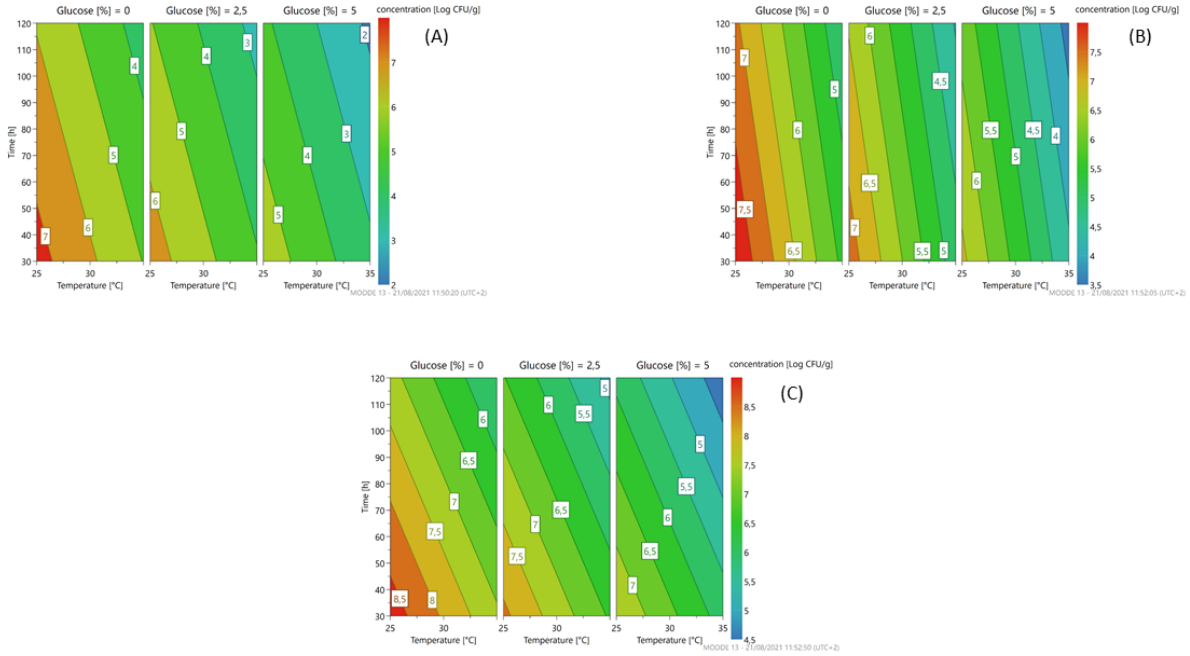
Table 3. Experimental condition for microorganism with an optimum at 30°C in vanilla without seeds

				***L. citreum* 4452**		***L. mesenteroides* 2194**		***W. minor* 4451**	
exp N.	Temp. (°C)	time (h)	glucose (%)	pH	Log cfu/g	pH	Log cfu/g	pH	Log cfu/g
1	25	30	5	6.91	6,80	6.73	6,30	6.69	7,42
2	35	30	0	6.98	6,15	6.78	5,09	6.75	7,05
3	25	120	0	6.99	7,11	6.95	6,78	6.92	7,14
4	35	120	5	6.90	3,00	6.95	3,00	6.88	4,16
5	30	75	2,5	6.77	3,00	6.80	6,27	6.71	6,22
6	30	75	2,5	6.70	3,00	6.77	6,14	6.71	6,28
7	30	75	2,5	6.66	3,00	6.83	5,65	6.80	7,12

In general, no growth was observed for all the strains. Moreover, condition n.4 seems where all the factors were set to the higher levels, seems to be the more stressful for these microorganisms, indeed the microbial load decreases, reaching values of about 3-4 Log cfu/g.

The response surface (Figure 6) gives us an indication for optimization similar to the previously described microorganism: less time, temperature, and concentration of glucose predicting a microbial growth of about 7-7,5 for *L. citreum* (6A) and *L. mesenteroides* (6B), while for *W. minor* it is possible to reach a higher microbial load about 8,5 Log cfu/g.

Figure 6. A response contour plot of concentration (Log cfu/g) of *L. citreum* 4452 (A); *L. mesenteroides* 2194(B); *W. minor* 4451 (C) in sample without seeds, with time and temperature as main factors and with glucose variation.



After completion of the experiments for the single strain cultures, we exploited the software's optimization function to predict the best growing conditions to use with co-cultures. To reach the highest microbial load (8 Log cfu/g), the software gave us the experimental conditions in which to operate. After the validation of these conditions, the co-cultures were made by combining two strains at a time, evaluating all possible combinations, combining strains with the same optimal growth temperature. For the *Lacticaseibacillus* genus, only the *L. casei* 2240 strain was considered, and not *L. paracasei*, because it had shown greater adaptability to the substrate. All the studied co-cultures are summarized in Table 4.

Table 4. Experimental conditions of fermentations with co-cultures in sample without seeds

Co-culture	Time (h)	Temperature (°C)	Glucose (%)	pH	Log Cfu/g
***L. rhamnosus* 1473 + *L. casei* 2240**	30	32	0.03	7,01	8,28
***L. rhamnosus* 1473 + *L. plantarum* 4932**	30	32	0.45	7,03	8,21
***L. rhamnosus* 1473 + *P. acidilactici* 3992**	30	32	0.03	7,08	8,18

***L. casei* 2240 + *L. plantarum* 4932**	30	32	0.06	7,03	8,18
***L. casei* 2240 + *P. acidilactici* 3992**	30	32	0	7,08	7,88
***L. plantarum* 4932 + *P. acidilactici* 3992**	30	32	0.06	6,86	7,92
***W. minor* 4451 + *L. mesenteroides* 2294**	30	25	0.06	6,96	8,13
***W. minor* 4451 + *L. citreum* 4452**	30	25	0.07	6,93	8,06
***L. citreum* 4452 + *L. mesenteroides* 2294**	30	25	0.08	6,98	7,65

Interestingly, in all the fermentations with co-cultures, it is recorded an increase in microbial load higher than in mono-cultures. The reason could be the synergistic effect of the microorganisms, and the choice of efficient fermentation conditions according to experimental modeling. In this set of experiments, no variation of pH was observed.

Fermentation of exhausted vanilla beans with seeds (S)

The seven experimental conditions for each strain combining time, temperature of fermentation, and addition of glucose were performed also for the sample with seeds (Table 5 and 6) for a total of 56 experiments. The aim was to evaluate if the different compositions of substrates led to modification in the measured responses. Also in this case, experimental conditions n. 5, 6, and 7 are biological replicates to account for natural variance and represent an intermediate condition for all experimental factors.

Table 5. Experimental condition for strains with an optimum at 37°C in vanilla beans with seeds.

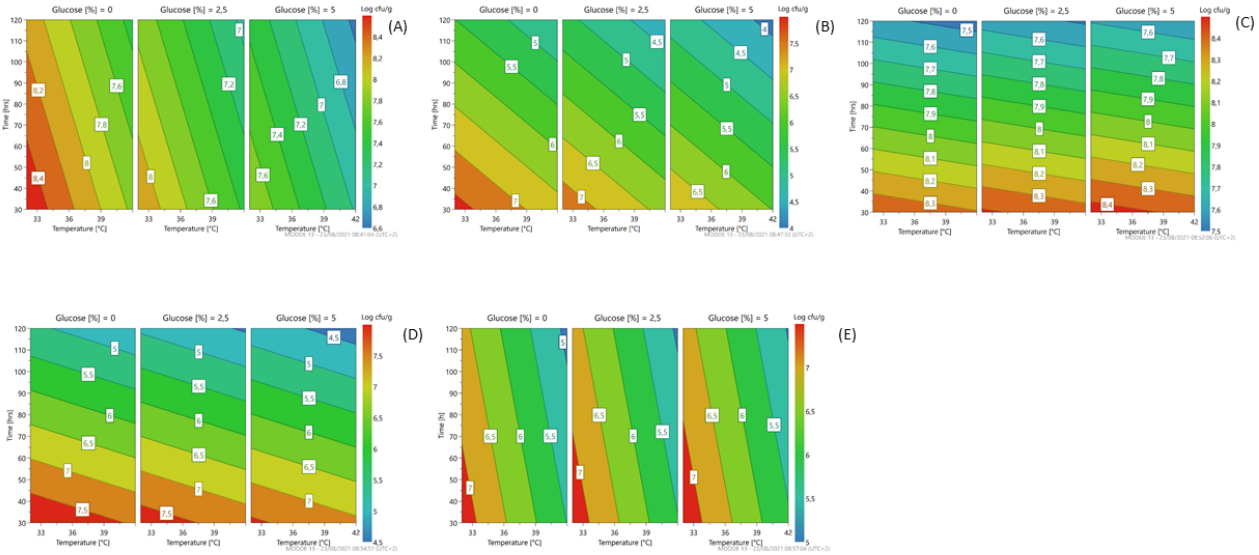
				***L. rhamnosus* 1473**		***L. paracasei* 4186**		***L. casei* 2240**		***L. plantarum* 4932**		***P. acidilactici* 3992**	
exp N.	Temp (°C)	time (h)	Glu. (%)	pH	Log cfu/g	pH	Log cfu/g	pH	Log cfu/g	pH	Log cfu/g	pH	Log cfu/g
1	32	30	5	6.89	7,27	7.02	7,15	6.52	8,32	6.66	7,32	5.26	7,18
2	42	30	0	6.92	7,25	7.10	6,88	6.66	8,18	6.71	7,18	4.88	5,57
3	32	120	0	6.83	7,62	6.94	5,69	6.73	7,40	6.54	4,83	5.01	6,46

4	42	120	5	6.70	6,04	6.97	4,00	5.31	7,39	6.60	4,00	6.10	4,00
5	37	75	2,5	6.21	8,07	7.18	5,00	6.13	8,01	6.57	6,38	6.46	5,61
6	37	75	2,5	6.11	8,31	7.06	5,70	6.23	8,13	6.66	6,38	6.53	4,98
7	37	75	2,5	6.33	8,32	6.82	5,81	6.48	8,22	6.71	6,61	6.47	5,56

Adaptation of the bacterial strains to these substrates was similar to that observed in samples without seeds. In particular, *L. rhamnosus* 1473 and *L. casei* 2240 are the best starters because they can grow, increasing the concentration of 1 Log cfu/g in almost all conditions for *L. casei* 2240, and in the optimal ones for *L. rhamnosus* 1473. Overall, regardless of growth, there is no lowering of pH except in condition n.2 during the fermentation with *P. acidilactici* 3992. The extreme condition (n.4) also in this case negatively affects the growth of *L. paracasei*, *L. plantarum*, and *P. acidilactici* as for the fermentation in vanilla beans without seeds.

The response surface (Figure 7), highlighted the same trend with respect to the fermentation in vanilla beans without seeds. In general, all the parameters could be set at minimum levels lead to a higher microbial load.

Figure 7. A response contour plot of concentration (Log cfu/g) of *L. rhamnosus 1473* (A); *L. paracasei 4186* (B); *L. casei 2240* (C); *L. plantarum* 4932 (D); *P. acidilactici* 3992 (E) in sample with seeds, with time and temperature as main factors and with glucose variation.



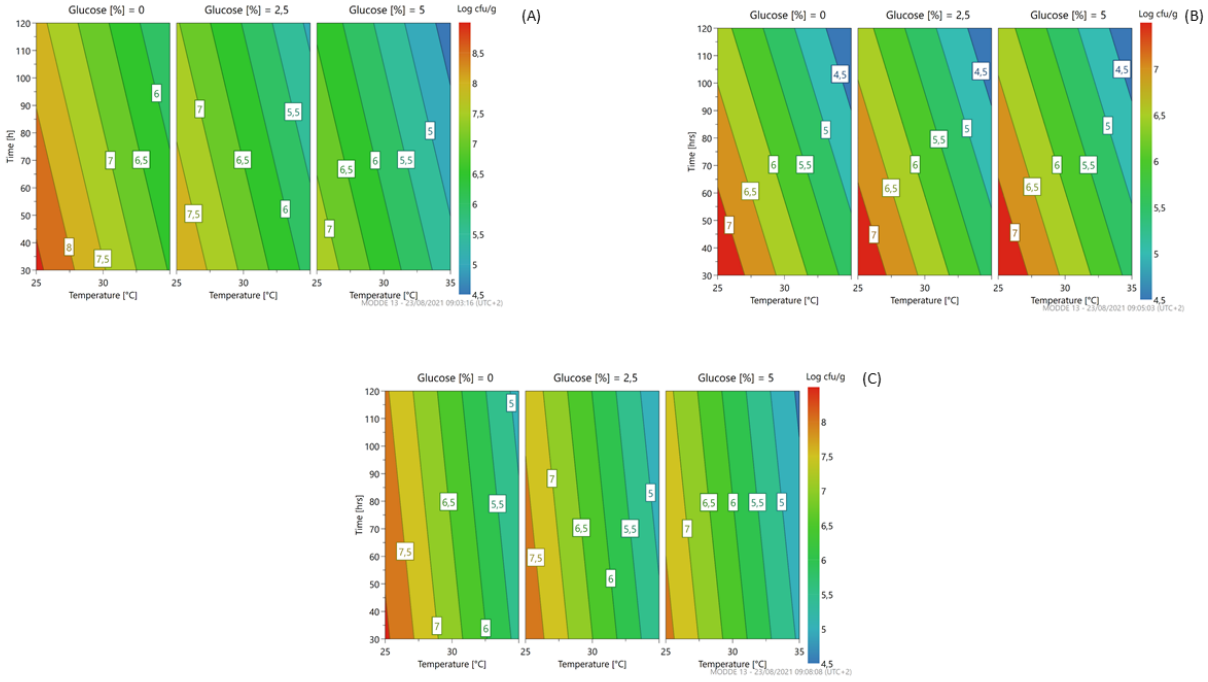
Regarding the strains with an optimum growth temperature of 30°C (Table 6), the best condition seems to be the one with a temperature of 25 °C. For *L. citreum* and *W. minor*, the concentration of sugar and fermentation time does not seem to influence growth, in fact there is a slight increase both in condition n.1 with the highest concentration of glucose, and in condition n.3 with the longer time. While for *L. mesenteroides,* the only condition where a slight increase is observed is condition n.1.

Table 6. Experimental condition for microorganism with an optimum at 30°C in vanilla beans with seeds

				***L. citreum* 4452**		***L. mesenteroides* 2194**		***W. minor* 4451**	
exp n.	temperature (°C)	time (h)	glucose (%)	pH	Log cfu/g	pH	Log cfu/g	pH	Log cfu/g
1	25	30	5	6,96	7,10	6,85	7,33	7,01	7,27
2	35	30	0	6,82	6,20	6,93	5,16	6,89	4,94
3	25	120	0	6,88	7,41	6,93	6,08	6,72	7,17
4	35	120	5	6,95	4,00	6,95	4,00	6,62	4,00
5	30	75	2,5	6,94	6,92	6,97	5,88	6,75	6,73
6	30	75	2,5	7,00	6,84	7,08	5,88	7,06	6,73
7	30	75	2,5	7,01	6,61	7,15	6,18	7,08	6,90

For all strains, the condition in which all parameters are set at the highest level is the most stressful. Also in this substrate, no variation of pH was recorded during the fermentation with these strains. Figure 8 resumes the response surface generated by the model in which it is possible to observe that *L. mesenteroides* (8B) is the only one that tolerates the addiction of glucose at high concentration, but maintains a bacterial load similar to the inoculum. On the other hand, the response surface suggests that *L. citreum* (8A) and *W. minor* (8C) could reach a higher concentration, about 7,5/8 Log cfu/g (red zone) with no addition of glucose and minimum temperature of fermentation set to 25°C.

Figure 8. A response contour plot of concentration (Log cfu/g) of *L. citreum* 4452 (A); *L. mesenteroides* 2194(B); *W. minor* 4451 (C) in sample with seeds, with time and temperature as main factors and with glucose variation



As described previously, the single-culture fermentations in vanilla without seeds were followed by co-culture fermentation in the same substrate (Table 7). The software's optimization function predicts the best growing conditions, to use in the validation step. To reach the highest microbial load (8 Log cfu/g), the software gave us the experimental conditions in which to operate. After the validation of these conditions, the co-cultures were made by combining two strains at a time, evaluating all possible combinations, combining strains with the same optimal growth temperature.

Table 7. Experimental condition of fermentation with co-cultures in sample with seeds

Co-culture	Time (h)	Temperature (°C)	Glucose (%)	pH	Log Cfu/g
***L. rhamnosus* 1473 + *L. casei* 2240**	30	32	0	7,04	8,08
***L. rhamnosus* 1473 + *L. plantarum* 4932**	30	32	0	7,05	8,00
***L. rhamnosus* 1473 + *P. acidilactici* 3992**	30	32	0	7,05	7,94

***L. casei* 2240 + *L. plantarum* 4932**	30	32	0	7,10	7,92
***L. casei* 2240 + *P. acidilactici* 3992**	30	32	0	7,09	8,02
***L. plantarum* 4932 + *P. acidilactici* 3992**	30	32	0	7,12	7,75
***W. minor* 4451 + *L. mesenteroides* 2294**	30	25	0	6,91	7,88
***W. minor* 4451 + *L. citreum* 4452**	30	25	0	6,95	7,86
***L. citreum* 4452 + *L. mesenteroides* 2294**	30	25	0	7,01	7,57

The growth is observed for all the co-cultures, also in this case reaching higher values than in fermentation with mono-culture. The best co-culture starters combination seems to be the ones composed by *L. rhamnosus* 1473 and *L. casei* 2240; *L. rhamnosus* 1473 and *L. plantarum* 4932; *L. casei* 2240 and *P. acidilactici* 3992.

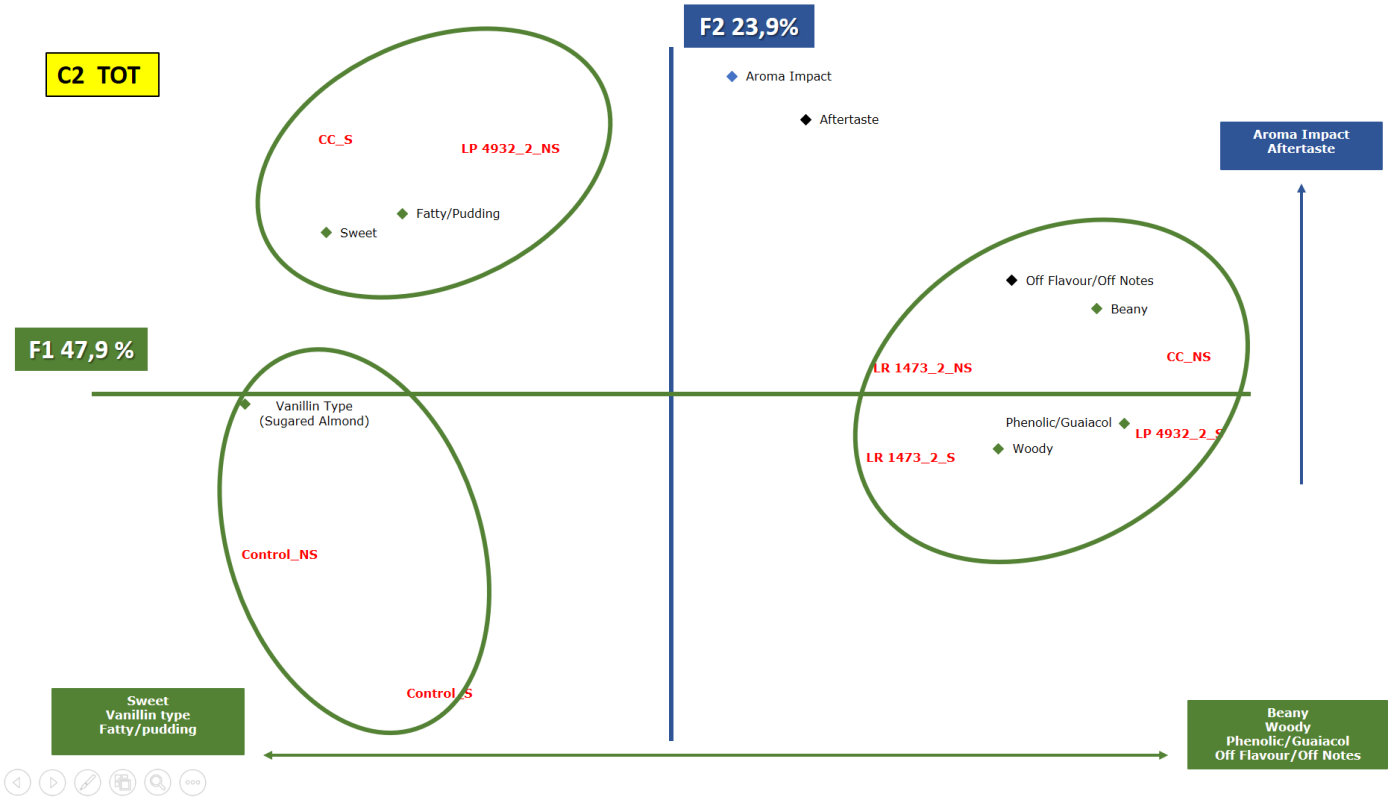
Sensory evaluation

The previously described screening led to the selection of strains combination for sensory evaluation. We selected the combination of strains *L. rhamnosus* 1473 and *L. plantarum* 4932 since these strains had shown a marked difference between the mono-culture (Table 2 and 5) and co-culture experiments (Table 4 and 7). We chose conditions 2 and 5 which are associated with the extreme and optimal conditions which led to a greater difference in performance

In the first tasting (Figure 4), unfermented and fermented samples with *L. rhamnosus* 1473 and *L. plantarum* 4932 and their co-culture were examined by panelists. Panelists were required to smell and taste the products and group them by similarity and/or difference, according to their personal criteria, associating each group with descriptive terms. To obtain more information on the parameters that could influence differences and similarities among the fermented samples in the two conditions (n.2 e n.5), the descriptors identified after tasting were used as variable vectors for Principal Component Analysis (PCA) reported in Figure 9 and 10.

Figure 9 represents the samples fermented with condition n.2 together with co-cultures. The first component (47,9 % of the information) is mainly characterized by descriptors such as sweet, vanillin type, fatty and pudding on one side, and beany, woody, phenolic, guaiacol, and off-flavors on the other side. The second component (23,9 % of the information) is characterized by higher aroma impact and aftertaste on the upper side.

Figure 9. Principal component analysis plot of the similarities and differences in the sensory profiling characteristics of exhausted vanilla beans prepared follow the condition n.2 of DoE and co-culture.



From the analysis, three groups were highlighted. The first group is the one composed of control samples, with and without seeds. These were judged by panelists as sweet and vanillin taste but with less aroma impact and aftertaste.

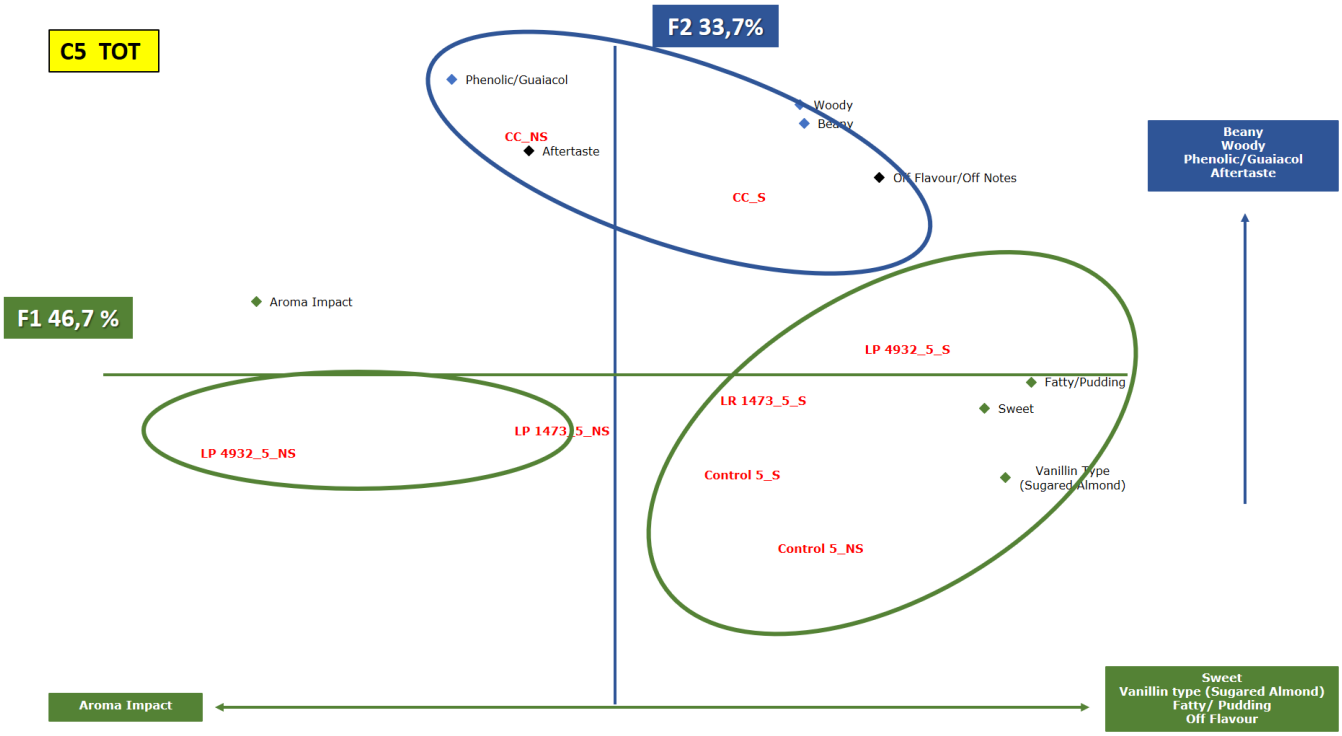
The second group is represented by co-culture with seeds and sample fermented with *L. plantarum* 4932 without seeds. These samples, as the previous ones, are described with vanillin and sweet notes, but in this case with a higher aroma impact.

The third group comprehends samples fermented with *L. rhamnosus* in both substrates (seeds and without), *L. plantarum* with seeds, and co-culture without seeds. These samples are described with beany, woody, phenolic and guaiacol notes.

Figure 10 shows samples fermented under condition n.5 together with co-cultures. The first axis (46,7 % of the information) is mainly characterized by aroma impact of the samples on one side, and descriptors such as sweet, vanillin type, fatty, and pudding on the other side. The second component (33,7 % of the information) is characterized by beany, woody, phenolic, guaiacol, and aftertaste.

In this condition, samples form three distinctive clusters as the previous, but a different trend is highlighted.

Figure 10. Principal component analysis plot of the similarities and differences in the sensory profiling characteristics of exhausted vanilla beans prepared follow the condition n.5 of DoE and co-cultures.



Substrates without seeds, fermented with monoculture, are in the same area of the graph characterized by aroma impact. On the other side, substrates with seeds fermented with the

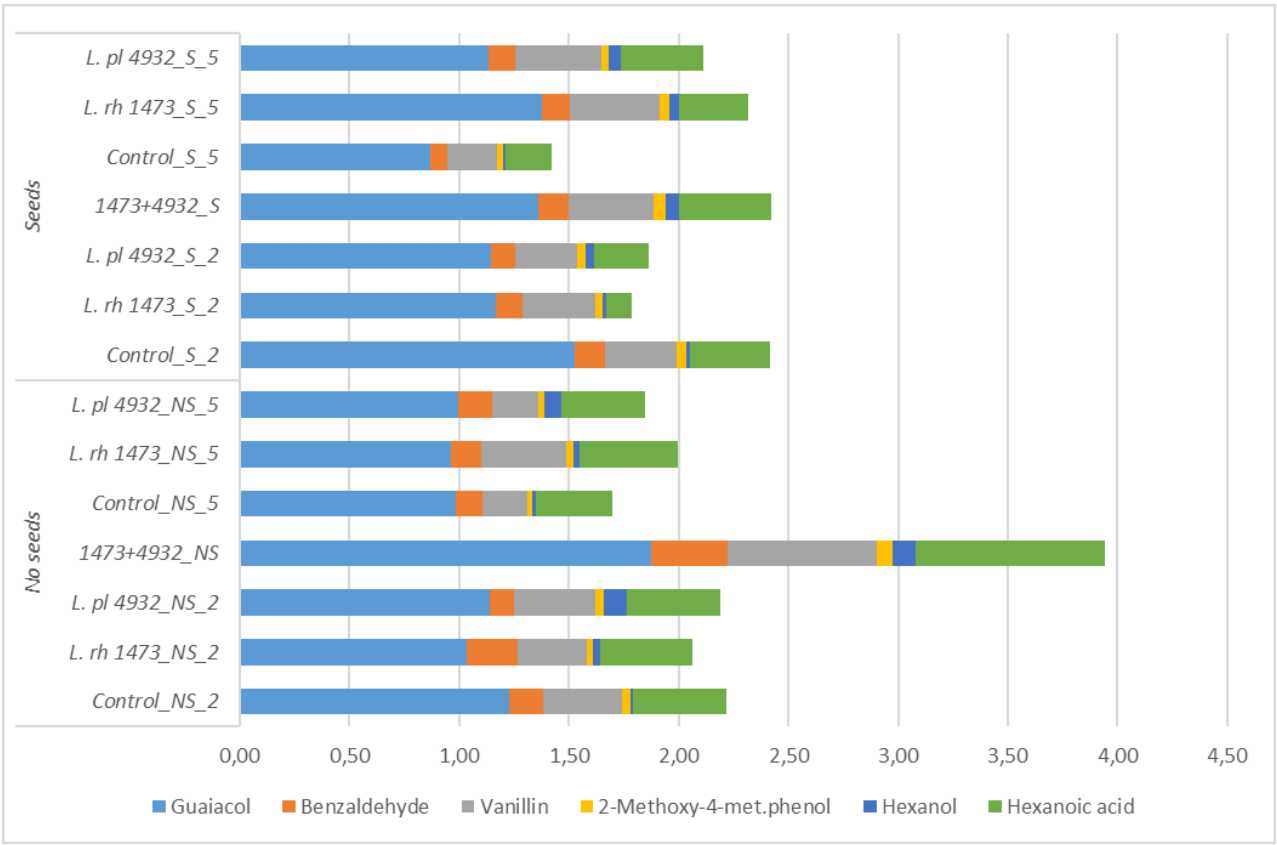
same strains lay on the opposite side of the figure, characterized by sweet, vanillin, pudding notes.

Co-cultures cluster separately in another region of the graph and are characterized by a beany, woody, guaiacol notes.

Volatile profile

From the analysis of volatile fractions, many compounds have been identified, but the attention has been focused on those that most characterize the aromatic profile of vanilla following the descriptors relating to the various organoleptic tests carried out. In particular, six volatile compounds were quantified after SPME-GC/MS analysis on fermented samples with *L. rhamnosus* 1473 and *L. plantarum* 4932 in mono and co-culture, and unfermented samples for both conditions (n.2 and n.5) together with the co-cultures. Figure 11 shows the concentration (µg/g) for each compound in all the tested samples.

Figure 11. Volatile compounds recorded in fermented and unfermented samples of exhausted vanilla beans. Concentrations are expressed in µg/g.



Starting from the control samples with and without seeds (unfermented), it is possible to observe that in general in condition n. 2 the concentration of compounds is higher than in condition n. 5, probably due to the increase in incubation temperature.

The concentration of guaiacol in the fermented samples with seeds appears to be slightly higher than in the control, in condition n.5. Conversely, substrates without seeds, in the same condition, remain stable. In condition n.2, the concentration of guaiacol of fermented samples decreases respect to the control both for substrates with and without seeds. Different trend is recorded for samples fermented with co-cultures where the concentration increases, in particular in substrates without seeds. Interestingly, the fermentation with co-culture in the vanilla beans without seeds leads to a significant increase of all the volatile compounds.

The concentration of benzaldehyde remains constant in almost all conditions except for samples without seeds, fermented with *L. rhamnosus* 1473 in condition n.2, and for the co-cultures.

Vanillin content increased after the fermentation by *L. rhamnosus*, in condition n.5, and by co-cultures in both vanilla substrates (with/without seeds).

2-methoxy-4-methyl phenol is higher in the sample fermented by the co-cultures (0.06-0.07 μg/g) respect to the others (0.02-0.04 μg/g).

In this study, we observed an increase of hexanol after fermentation with *L. plantarum* and co-culture in vanilla without seeds, while in the other substrate also after fermentation with *L. rhamnosus,* especially in condition n. 5.

The concentration of hexanoic acid deeply decreased after fermentation with *L. rhamnosus* in substrates with seeds, in condition n.2. While an increase in the sample without seeds especially in the co-culture sample was recorded.

Discussion

The aim of this study was to verify the fermentability of the exhausted vanilla beans to add value to the by-product, obtaining high value molecules, and evaluate the differences between samples with and without seeds for their possible application.

Statistical modeling using DoE has been a preferred approach when it comes to studying multiple factors. The DoE and the Response Surface Methodology (RSM), in particular, have been of great industrial benefit in simplifying multifactorial experimental design. Indeed, we explored multiple conditions to study the different behavior of LAB strains and find the right starters. Moreover, to better understand the modifications made by microorganisms we conducted a preliminary investigation on sensory profile, to evaluate the possible use in the food company. We applied DoE to understand the best fermentation condition in which to operate, leading to the selection of conditions that are far from the optimal cultivation conditions for the microorganisms. The current experimental design approach points towards setting the parameters at the minimum levels for all the selected conditions: fermentation operated for less hours, at temperatures below the optimal and without glucose addition.

Biodiversity among LAB, species- and strain-dependent, was observed as the strains showed different adaptation to the vanilla substrate. Notably, no significant differences were observed when bacteria were inoculated in the samples with and without seeds, except for the strains *P. acidilactici* 3992, *L. plantarum* 4932, *L. paracasei* 4186, and *L. citreum* 4452. Indeed, cultivation in the substrate without seeds led to a decrease of microbial concentration, which is more evident in conditions 4, 5 6, and 7, all characterized by sugar addition and an incubation time from 75 to 120 hours.

On the other hand, the strains *L. rhamnosus* 1473 and *L. casei* 2240 showed good adaptability to both the substrates, increasing the microbial load of about 1 Log cfu/g in almost all conditions. The similarities in the growth ability may be linked to the genotype-phenotype relationship. Moreover, the ability of *Lacticaseibacillus* isolates to ferment different types of vegetable substrates is recently reported for other plant substrates (Hadj Saadoun, Ricci, et al., 2021; Ricci, Cirlini, et al., 2018; Spaggiari et al., 2020).

Interestingly, the fermentation condition n.2 of DoE often leads to decrease in pH, but only in strains with optimum growth temperature at 37°C. Generally, the decrease of pH is associated with an increase in microbial load, but in this case, it was observed even when

there is no growth or cell lysis. This could be explained as a mechanism in response to stress environment since the temperature was set at maximum level.

LAB co-cultures are not widely studied even if they seem advantageous respect to the mono-cultures due to the synergistic action of the metabolic pathways of the strains involved (Chen, 2011). Strain formulation for co-culture experiments was selected according to the predictive function of the DoE model, combining the strains that are expected to work best at the same operating conditions.

In this study, we demonstrated that the selected strains show increased growth in exhausted vanilla beans, of about 1 Log cfu/g in all cases respect to the single cultures. This is in agreement with previous study where the two types of cultivation were compared (Hadj Saadoun, Calani, et al., 2021).

Differences in substrates composition did not affect microbial growth, but it affected the aromatic notes and the pH, which did not change in the presence of seeds. During the sensory panel test, the panelists highlighted a different profile for the two substrates. In particular, samples without seeds are described as less tasty, with notes of beany, woody, and phenolic. Samples with seeds were evaluated as sweet with vanillin, fatty, pudding notes.

During the sensory panel test, differences between the fermented samples could be referred to the fermentation conditions and strains used. Notably, also strains that did not show a significant increase in microbial concentration, like *L. plantarum*, showed the ability to modify the sensory perception of the fermented sample. Variations in the volatile profile in the absence of growth in inoculated substrates were already observed (Ricci, Levante, et al., 2018), and highlight the importance of a combined approach, including sensory profile evaluation, for the evaluation of strains to be applied in industrial fermentation.

The volatile profile of the substrates, with or without seeds, were affected by fermentation differently, according to the selected conditions. Quantitative analysis showed that the effect of fermentation on the concentration of the principal volatile compounds was not greatly influenced, except for the co-cultures, which showed an increase in the concentration of hexanoic acid, hexanol, and guaiacol. Accumulation of these compounds could be due to

an interaction of the metabolic pathways of the strains, as confirmed by the higher microbial load observed in these samples at the end of fermentation. These differences resulted in a distinctive sensory perception, that drove the clustering of samples in the PCA analysis.

Conclusion

The results presented in this work underline the effectiveness of the DoE approach for the screening of combinations of strains and conditions for fermentation. The integration with the sensory and instrumental analysis of the samples allowed us to have a broader view of the effect of fermentation in this substrate.

Furthermore, there is an indication that the use of combinations of strains in co-culture for fermentation can lead to the development of distinctive notes in the final product. Investigation of the metabolic network occurring during the co-cultivation of the strains would be required to expand the knowledge on microbial interactions. In agreement with the obtained results, solid state fermentation can be considered a promising approach for the recovery and valorization of vanilla by-products. Further studies will be oriented towards selecting the appropriate applications for these fermented by-products as ingredients or flavouring agents in food products

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Chapter 3

A critical review of intrinsic and extrinsic antimicrobial properties of insects

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ABSTRACT

Background: Sustainable nutrition and food security are central topics in the current global agenda. Insect production represents a promising food source that has received growing attention in the food and feed industry due to its high nutritional value and positive effect on animal health.

The resilience of insects to infections by different parasites indicates they are an excellent type of biomass for the extraction of various antimicrobial compounds.

Scope and approach: The antimicrobial potential of insects is illustrated through a description of the different sources of antimicrobial compounds. Distinctions between intrinsic and extrinsic insect antimicrobials are presented in terms of their constituent components and metabolites produced. An updated overview of potential applications in food and feed sectors is presented to identify key reasons to invest in this food source and to stimulate further research in this field. The main aspects supporting the development of this industry in the future are summarized.

Key findings and conclusions: Processing extracted insect fractions will lead to the development of new application possibilities and an increase in the edible insect sector value. The possibility of producing new generation antimicrobials from a sustainable supply chain involving insect rearing contributes to green economy policies and reduction of antibiotic resistance.

Introduction

Sustainable nutrition and food security are the central focus of the global agenda supported by the United Nations Sustainable Development Goals (United Nations, 2015). One of the aims is to feed approximately 9 billion people by 2050 and provide safe and nutritious food for everyone. Many factors pose challenges to global food safety including the increasing complexity of food supply chains, environmental constraints, a growing aging population, and changing consumer choice and food consumption patterns (King et al., 2017). Maintaining a safe food supply is challenging due to the emergence and re-emergence of foodborne pathogens (Flynn et al., 2019).

The World Health Organization (WHO) estimates that unsafe food causes 600 million worldwide cases of foodborne diseases and 420,000 deaths annually (World Health Organization, 2021). Pregnant women, infants, young children, and the elderly are the most susceptible, however, all individuals are at risk (Flynn et al., 2019).

The impact of food safety outbreaks has potentially drastic health outcomes and impacts food business (Hussain & Dawson, 2013) which may damage a particular food industry sector on a short and long term. Therefore, food companies need to improve the microbiological safety of foods that are produced locally and internationally (Hussain & Dawson, 2013).

Infectious diseases caused by microorganisms such as bacteria, viruses, and fungi are one of the major problems for human and animal health. Treatment involves particular antimicrobials with different mechanisms of action and pharmacological properties. Antibacterial agents act on structural features of prokaryotic cells and interrupt metabolic processes, while antiviral agents typically affect viral nucleic acid synthesis, and antimycotics often target key components of replicative or biosynthetic fungal pathways (Singh et al., 2021). Antimicrobial agents are frequently used in medicine, agricultural production, veterinary applications, and food processing due to their broad spectra of activity (Hayashi et al., 2013). The antimicrobials used in these fields are either chemically synthesized compounds such as quinolones, or naturally derived products from microbes,

plants, and animals (Fu et al., 2016; Roller, 1995). There is a growing interest in insect antimicrobial compounds due to their unique properties.

The increasing literature regarding natural antimicrobial compounds in the last decades shows a growing interest and demand for their production in the coming years. This review carefully illustrates the different antimicrobial sources, differentiates intrinsic and extrinsic antimicrobials derived from insects in terms of their constituent components and metabolite production, provides an updated overview of the potential applications in food and feed sectors, and highlights key reasons to invest in this source to inspire the scientific community. This will lead to the utilization of insect antimicrobials to extend the market size of the sector, support the circular economy, contribute to environmental sustainability, and reduce antibiotic resistance.

Antimicrobials: a plethora of natural sources

This section will provide a brief overview of the different antimicrobial natural sources from microorganisms, plants, and animals. Microorganisms are an invaluable and well-known resource of antibacterial compounds. The first antibiotic was discovered from *Penicillium sp.* fungus in 1928. Other fungi are also well-known producers of biologically active antibiotics and secondary metabolites (Skellam, 2019). Bacteria often inhibit undesirable bacterial growth by synthesizing bacteriostatic or bactericidal catabolism end-products (organic acids, ethanol, hydrogen peroxide, and diacetyl), and bacteriocins (Sun et al., 2015). Bacteriocins are specific proteinaceous antibacterial substances that are active against closely related bacteria (Reis et al., 2012). The most well-known bacteriocins are probably those produced by lactic acid bacteria since their synthesis, structure, mechanism of action and classification is previously described (Juturu & Wu, 2018). Bacteriocins are cationic peptides with hydrophobic or amphiphilic properties and bioactivity typically targeting the bacterial membrane (Cavera et al., 2015). Recent research has focused on the use of microorganisms to ferment plant by-products such as peels, seeds, pulps, husks of fruits and vegetables to obtain natural antimicrobials (Gyawali & Ibrahim, 2014; Ricci et al., 2019). Plants and their natural antimicrobial components from some herbs and spices have been used since ancient times with 3,000 essential oils shown to exhibit antibacterial activity. For

instance, cinnamon oil is one of the first plant components discovered for its antimicrobial effects (Gyawali & Ibrahim, 2014). Different phytochemical compounds with similar antibacterial properties were later discovered including phenolic compounds, alkaloids, quinones, tannins, and plant antimicrobial peptides (Hintz et al., 2015). The antibacterial and antifungal effects of herb and spice extracts from basil, oregano, and thyme are attributed to their phenolic components (Sakkas & Papadopoulou, 2017). Generally, these compounds are produced by secondary metabolism defense pathways. Several different mechanisms of action were proposed depending on their chemical structures, including bacterial cell membrane alteration (Wang et al., 2018), and hemolysis (Du et al., 2018).

Lysozymes are a broad class of enzymes in the animal kingdom that show specific hydrolytic activity against bacterial peptidoglycans of cell walls (Ragland & Criss, 2017). Antimicrobial peptides from vertebrate and invertebrate species improve the immune response against pathogens. The list of discovered antimicrobial peptides is constantly increasing. Some examples include pleurocidin from winter flounder, lactoferrin derived milk peptide, and defensin from mammalian epithelial cells (Brogden, 2005). They typically interact with the bacterial membrane and disrupt its integrity with their mechanism of action depending on their primary structure and higher-order structure (Lei et al., 2019).

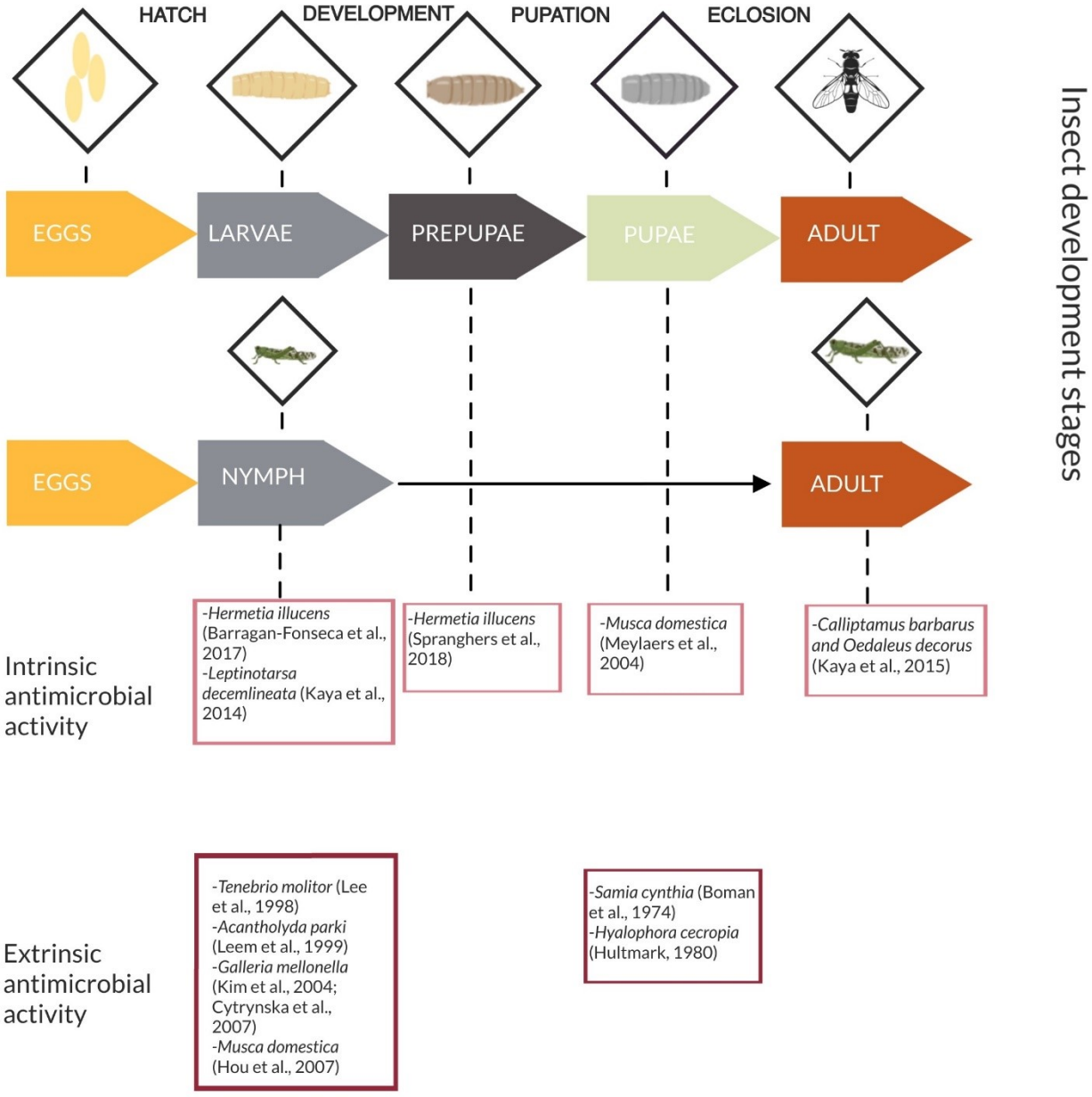
The high degree of biodiversity in the animal kingdom and the habitual exposition to infections suggest that further research should be conducted to discover new antimicrobials from non-conventional animals. In particular, invertebrates such as insects represent approximately 75% of all animal species and portray a largely unexploited source of potential antimicrobials (Ali et al., 2018). For this reason, we believe that this field of study is timely and topical.

Antimicrobial activity of insects

Interaction between bacteria and insects has recently attracted interest in the scientific community. On one hand, some bacteria that colonize the insect body produce metabolites that are selectively toxic to invertebrates such as DNA-intercalating compounds (Ho et al., 2020). On the other hand, the insect gut microbiota produces a wide range of metabolites in defense against pathogenic bacteria (De Smet et al., 2018).

Insects and their extracts are widely used in medicine in many parts of the world for the treatment of various human diseases (Ratcliffe et al., 2011). The first report of insect antimicrobial properties showed inducible antibacterial activity in the hemolymph of silk moth (*Samia cynthia*) pupae (Boman et al., 1974). New discoveries have since shown the arsenal of antimicrobials produced during their growth stages: egg, larva, prepupae, pupae, and adult individual (Figure 1).

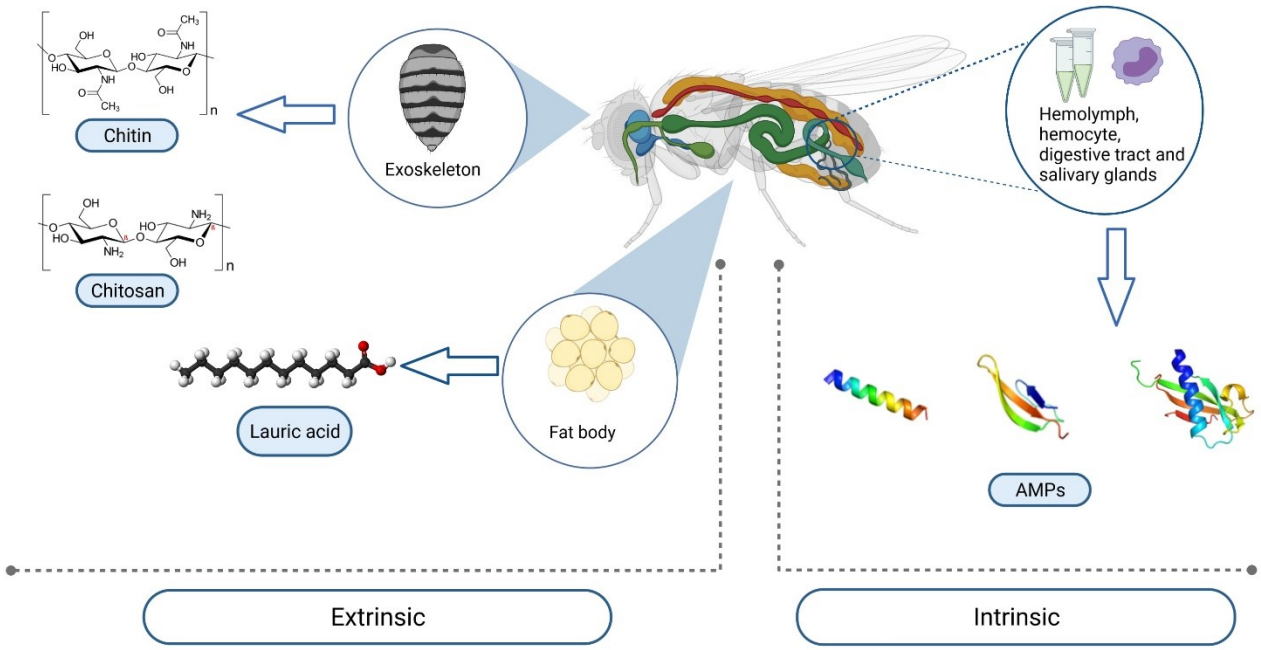
Figure 1. Schematic representation of antimicrobial compounds found in different insects at diverse stages of development.



Metamorphosis promotes the exploration of different ecological niches and exposure to different parasites (Critchlow et al., 2019). Hatching involves the exit of larva from the chorion and designates a shift towards larval development into prepupa. During this phase, the main roles of the growing insect are feeding and surviving in an often hostile environment, and preventing microorganism invasion which occurs by oral ingestion or entry through the cuticle. The innate immunity of insects is the reason for their diffusion and evolutionary success (Sheehan et al., 2020). Two lines of defenses against pathogens exist i) the cuticle, a complex barrier mainly comprised lipid and chitin with antimicrobial properties designed to prevent or retard the entry of pathogens; ii) the hemolymph, analogous to mammalian blood and is involved in transport of nutrients, waste products, and signal molecules, and contains cellular and humoral defense mechanisms responding to a variety of microorganisms (Vallet-Gely et al., 2008). Cellular defense is represented by immune cells (hemocytes) which kill pathogens via phagocytosis, melanization and lysis (Dolezal et al., 2019). Humoral defense contains numerous antimicrobial peptides (AMP)/polypeptides that work synergistically to destroy invading microorganisms (Vallet-Gely et al., 2008).

The two lines of defense are defined as intrinsic and extrinsic according to the constitutive or inducible nature of the antimicrobial compounds (Figure 2).

Figure 2. Schematic representation of the insect immune system. The two lines of defense containing intrinsic and extrinsic antimicrobial activity are represented by body components and humoral response, respectively.



Intrinsic defense is represented by constitutive molecules of the body that possess antibacterial activity, while extrinsic defense is related to inducible metabolites synthesized in the body to counteract infections. Different examples of antimicrobials attributable to these two categories are mainly reported from three orders of insect species including *Galleria mellonella* (Lepidoptera), *Drosophila melanogaster* and *Hermetia illucens* (Dipterae), and Apis mellifera (Hymenoptera) (Figure 1, Critchlow et al., 2019).

Intrinsic antimicrobial activity: body composition

Insects contain a good source of proteins, lipids, vitamins, and minerals and are considered a potential alternative as food and/or feed (Sogari et al., 2019). Their body composition differs according to the species, developmental stage, and growth substrate (Nowak et al., 2016). For example, *H. illucens* larva are rich in protein, the prepupa have a high lipid content, while the pupa contains elevated chitin (Smets et al., 2020). This species metabolizes a large proportion of fatty acids into lauric acid (Barragan-Fonseca et al., 2017; Hadj Saadoun et al., 2020a; Hadj Saadoun et al., 2020b). Lauric acid (C12:0) has antimicrobial activity

against gram-positive bacteria such as *Clostridium perfringens* while being less effective against gram-negative bacteria. Its mechanism of action is unclear, but it is known to interfere with bacterial cell signal transduction and gene transcription following cell membrane destruction (Dayrit, 2015).

Chitin is a naturally abundant linear polymer composed of N-acetyl-glucosamine and is the main exoskeleton component in insects and crustacean shells. Chitin may be converted into chitosan chemically or enzymatically by deacetylation which increases its antimicrobial activity. Chitosan is the more potent antimicrobial for two reasons: i) it possesses many positively charged amine groups which may interact with components of the bacterial cell surface (lipids, proteins, carbohydrate); ii) higher solubility (Khattak et al., 2019). Chemical synthesis of chitosan has several disadvantages including high-cost production, inconsistent molecular weight, low degree of acetylation, and environmental impact (Philibert et al., 2017). Alternatively, biological deacetylation is possible through microbial fermentation of chitin from shrimp shells (Zhang et al., 2017).

Chitosan extracted from the grasshopper species, *Calliptamus barbarus* and *Oedaleus decorus,* has antimicrobial activity against gram-negative bacteria (Kaya et al., 2015). Similarly, the greatest chitosan antimicrobial activity from *Leptinotarsa decemlineata* adults and larvae is against the gram-negative bacteria, *Salmonella enteritidis* and *Bacillus subtilis* (Kaya et al., 2014). The mechanism of chitin and chitosan inhibition against bacteria is incomplete and is influenced by several factors (Khattak et al., 2019; Kong et al., 2010). Two hypothetical modes of action are proposed: i) attraction of opposite charges of chitosan with the bacterial membrane; ii) RNA and protein inhibition due to chitosan diffusion in microbial cells (Khattak et al., 2019).

Chitin and chitosan are mainly derived from crustacean exoskeletons (Philibert et al., 2017), however, insects represent a valuable alternative source of these two molecules. Although fatty acids (particularly lauric acid) and chitin/chitosan are the most researched intrinsic insect antimicrobial molecules, other compounds have been identified. For example, 1-lysophosphatidylethanolamine (C16:1) is a constitutive antimicrobial compound in the

Musca domestica (housefly) membrane that is effective against gram-positive bacteria (Meylaers et al., 2004).

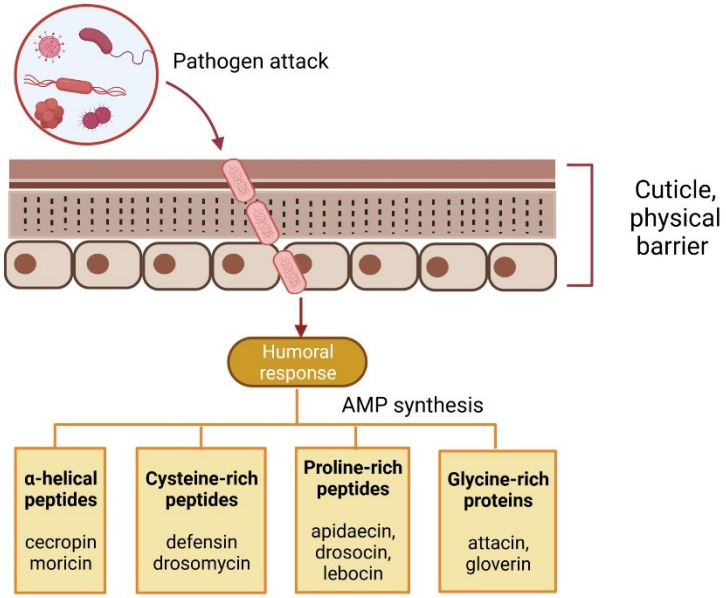
Extrinsic antimicrobial activity: AMP production

The humoral response of insects involves the synthesis of a wide range of AMPs in the fat body, hemocytes, digestive tract, salivary glands, and the reproductive tract. Pathogen recognition by pattern-recognition receptors leads to AMP release into the hemolymph (Buonocore et al., 2021). These peptides have strong antibacterial and antifungal activities comparable to similar compounds present in vertebrates and plants. The first insect AMP purified from *Hyalophora cecropia* pupae (Hultmark et al., 1980) led to the discovery of new AMP from arthropods and is currently a hot topic (Feng et al., 2020).

The common feature of AMPs is their small size (below 100 amino acids) and their positive charge. Their mechanism of action depends on the type of AMP and the target pathogen. AMPs may interact with the microbial membrane surface, alter the permeability and induce cell lysis, or enter the cell and damage bacterial components such as DNA and RNA (Sheehan et al., 2018; Wu et al., 2018). The charge, sequence, conformation, and structural properties (hydrophobic and amphipathic) are important for the antibacterial activity of AMPs (Wu et al., 2018). AMPs inhibit a wide range of bacteria and/or fungi, and may present an alternative to fight antibiotic-resistant bacteria. There is a strong recent interest in the use of insect AMPs in livestock production as food supplements (Józefiak & Engberg, 2017; Wang et al., 2016).

Insect AMPs may be classified into four families based on their amino acid composition and secondary structures: (i) α-helical peptides (cecropin and moricin), (ii) cysteine-rich peptides (defensin and drosomycin), (iii) proline-rich peptides (apidaecin, drosocin, and lebocin), and (iv) glycine-rich proteins (attacin and gloverin) (Figure 3).

Figure 3. The humoral response following pathogen attack involves the synthesis of various antimicrobial peptides (AMPs).



Cecropins, defensins, proline-rich peptides, and attacins are the most common insect AMPs, while gloverins and moricins have only been isolated from Lepidoptera (Yi et al., 2014). Cecropins represent the most abundant linear α-helical AMPs in insects with antimicrobial activity against gram-positive and gram-negative bacteria, antifungal activity, and anti-inflammatory activity (Manniello et al., 2021) with their mode of action involving bacterial cell membrane lysis and inhibition of proline uptake (Wu et al., 2018).

Insect defensins are the largest AMP group isolated from several orders including Diptera, Hymenoptera, Hemiptera, Coleoptera, Lepidoptera, and Odonata. These peptides have a greater effect against gram-positive bacteria compared with Gram-negative bacteria (Yi et al., 2014). Meanwhile, defensin from *G. mellonella* larvae has antifungal activity (Kim et al., 2004).

Proline-rich peptides are mainly active against gram-negative bacteria although lebocins that were first isolated from the silkworm *Bombyx mori* have antimicrobial and antifungal activity. Apidaecins isolated from *A. mellifera* are also involved in humoral defense against microbial invasion (Manniello et al., 2021).

Glycine-rich proteins are mainly represented by attacins classified as acidic attacins and basic attacins. They are generally active against gram-negative bacteria and gram-positive bacteria (Buonocore et al., 2021).

Many new AMP from insects were identified after bacterial infection. For example, eight peptides from the hemolymph of *G. mellonella* larvae have greater selectivity against gram-positive bacteria compared with gram-negative bacteria, although one peptide has dual selectivity (Cytryńska et al., 2007). Antimicrobial peptides inhibiting foodborne pathogens were isolated from *M. domestica* larvae (Hou et al., 2007). Similarly, an antibacterial protein from *Tenebrio molitor* larvae and a defensin-like peptide (DLP4) from *H. illucens* larvae have activity against gram-positive bacteria, particularly methicillin-resistant *Staphylococcus aureus* (Lee et al., 1998; Park et al., 2015).

Other antibacterial substances were isolated from immunized insect extracts. For example, N-beta-alanyl-5-S-glutathionyl-3,4-dihydroxyphenylalanine from adult *Sarcophaga peregrina* and p-hydroxycinnamaldehyde from *Acantholyda parki* larvae are inducible compounds with antibacterial activity (Leem et al., 1999; Park et al., 2014). Although most insect AMPs and other antimicrobial compounds described above are induced with microorganisms or microbial extracts, AMPs may be constitutively produced in insects, such as termicin from hemocyte granules and salivary glands of *Pseudacanthotermes spiniger* or drosomycin in the salivary glands and female reproductive tract of *D. melanogaster* (Lamberty et al., 2001; Uvell & Engström, 2007).

Applications of insects as potential antimicrobial sources in the food and feed sector

The utilization of insects as food and feed is receiving increasing attention from a range of disciplines thanks to their unique characteristics and this has generated a rise in the number of scientific studies (Payne et al., 2019). The high nutrient value of insects as an alternative source of protein, fat, and several other compounds including micronutrients and chitin may help to reduce food hunger, food insecurity, malnutrition (Borrelli et al., 2021), and has redefined the frontiers of the practice and the study of entomophagy.

It is difficult to generalize the nutritional value of insects considering their large biodiversity which varies according to the development stage and feeding regimen (van Huis et al.,

2013). Nevertheless, the protein and lipid levels across a broad spectrum of insect species are significantly high with the protein content in many insects above 60% of the dry mass. These proteins are highly digestible (77% -98%) with essential amino acid scores of 46% - 96% (Belluco et al., 2013). Insect fat content ranges from 12.97% to 24.7% with high levels of essential fatty acids, especially linoleic and linolenic acid. Moreover, chitin is linked to improved human gut microbiology and gastrointestinal health. Finally, many insects are rich in micronutrients including magnesium, iron, zinc, and copper (Selenius et al., 2018; Zielińska et al., 2015).

This nutritional profile of insects suggests that they have the required characteristics for human consumption as whole insects and as insect-derived fractions. Consequently, insects are often processed by fractionation to obtain high-grade material including oil and protein extract which are evaluated by their sensorial features (color and aroma) and added as an ingredient in food formulations (Lakemond et al., 2019; Reverberi, 2020). Insect extract is a growing industry due to increasing demand of high protein food for sports nutrition, dietetic food, and other food supplements (Placentino et al., 2021).

Insects fall within the novel food legislation by the European Union (EU) (European Commission 2015a). Whole edible insects and their derived ingredients may only be lawfully placed on the EU market after safety assessments and authorizations (Ojha et al., 2021). Currently, the European Food Safety Authority (EFSA) assessed that two edible insect species are safe for human consumption: dried yellow mealworm (*T. molitor* larva) (EFSA Panel on Nutrition, Novel Foods and Food Allergens (NDA), 2021b) and migratory locust (*Locusta migratoria*) (EFSA Panel on Nutrition, Novel Foods and Food Allergens (NDA), 2021a).

Insects are a natural feed source for animals (poultry) besides being a good protein source for monogastric animals (Menozzi et al., 2021). The latest entrepreneurial and regulatory developments favor the opportunities for employing insects as feed (Cadinu et al., 2020; Sogari et al., 2019). The EU feed legislation has allowed the use of insects in feed for pets and aquaculture animals since July 2017, while live insects are permitted under national legislation in certain EU Member States for fish, poultry, and pig (Menozzi et al., 2021). More

recently, the EU Member States voted positively on a draft regulation aimed at enabling the use of processed insect proteins in poultry and pig nutrition (Sogari et al., 2021).

The positive effects on animal health using insect AMP as feed supplement include improvements in growth performance, nutrient digestibility, intestinal morphology, and animal gut microbiota in pigs and chickens (De Marco et al., 2015; Wen & He, 2012; Yoon et al., 2013). Moreover, the oils extracted from insects based on lauric acid and its derivatives have antimicrobial effects in vitro and in vivo with a reduction in the growth of *Yersinia enterocolitica*, *Campylobacter coli*, and Enterobacteriaceae resulting in an improvement in the colonization of the intestinal microbiota by healthy bacteria (Dabbou et al., 2020; Zeiger et al., 2017). *H. illucens* prepupae fat is proposed as an alternative antimicrobial agent for use in feed to reduce the need for antibiotics (Spranghers et al., 2018).

It is well known that antibiotics are widely used in livestock for disease prevention and growth promotion, however, their inappropriate and expanding use in the last decade has caused food safety threats with the emergence of antibiotic resistance (Flynn et al., 2019). Consequently, the search for antibiotic alternatives is an urgent challenge to maintain animal production performance and animal health and welfare (Józefiak & Engberg, 2017). This is an issue that concerns animals and humans because many common foodborne zoonotic bacterial pathogens are antibiotic resistance carriers. The increasing antimicrobial resistance in manure-amended soil may enter the food chain resulting in the transmission of antibiotic resistance genes into the human microbiome (Zhang et al., 2019). Global solutions to reduce antimicrobial intake in animals by up to 80% include regulatory caps, price interventions, and reductions in meat consumption (Van Boeckel et al., 2017). In addition, the antimicrobial value of insects described in this review may be used as food and feed additives. Moreover, constituent components and metabolites produced from insects are not resulting in the development of natural bacterial resistance. It is well established that AMPs may bypass the common resistance mechanisms for three main reasons. First, AMPs have several antimicrobial target sites compared with a single target site for conventional antibiotics and typically act on the bacterial membrane rendering the cell unviable. It is “costly” for bacteria to counteract this AMP activity since it requires

membrane redesign by introducing genetic mutations. Second, AMPs often have multiple mechanisms preventing bacterial survival (Wang et al., 2016). Third, AMPs are modulators of innate immunity (Li et al., 2021; Wang et al., 2016). Similarly, it is more difficult for susceptible pathogens to develop resistance to medium-chain fatty acids such as lauric acid which target the membrane (Jackman et al., 2020).

A less explored application of insect or insect-derived products is biopreservation involving increasing the shelf life and/or safety of food products by using natural or controlled microflora or antibacterial products. Chitosan has attracted growing attention as a food preservative due to its versatility, nontoxicity, biodegradability and biocompatibility (Hu & Gänzle, 2019). This natural biopolymer coating material is suggested to preserve the quality of fresh food like fruits, vegetables, and meat and to extend their shelf life (Haghighi et al., 2020; Kumar et al., 2020). The chitosan market size is expected to considerably grow given its wide range of applications. There are currently three main sources of chitin/chitosan: crustaceans, fungi and insects, with the exoskeleton of crustaceans derived from waste streams of marine food industries currently the most exploited. The increasing insect rearing facilities worldwide may promote insects as a primary source of chitin/chitosan (Hahn et al., 2020) since it contains less calcium carbonate (<6%) compared to crustaceans (30–50%) and has an easier, environmentally friendly extraction method potentially leading to sustainable production throughout the year (Cadinu et al., 2020). AMPs may be potentially used as general food preservatives to inhibit undesired microorganisms since an AMP extracted from housefly (*M. domestica*) has similar effectiveness as a preservative in orange juice compared with conventional preservatives (Hou et al., 2007).

Despite significant efforts in the use of insects or their products in biopreservation, studies on the exploitation of antimicrobials from insects as preservatives are limited. Considering their potential implications (Tiwari et al., 2009), we believe that this review will spark the emergence of a novel field of research.

Why focus on insects in the future?

Insect production has emerged as a crucial stepping stone to provide safe and high-quality raw materials, ingredients, and products for large-scale food and feed applications

considering the raised awareness of environmental issues linked to the food system and the need for a circular economy (European Commission, 2015b). Three main key points are highlighted for the development of the insect sector: i) extension of the insect extract market size, ii) sustainable food production, iii) reduction of antibiotic resistance.

i) Extension of the insect extract market size

Despite edible insect production progressively gaining importance, this sector is fragmented based on the final products. The companies that produce and sell edible insects are currently represented by small and medium-sized enterprises and are mainly limited to *T. molitor* and *Acheta domesticus* species (Payne et al., 2019; Pippinato et al., 2020).

The major challenges for the insect industry are reductions in cost price and the upscaling and automation of rearing (Lakemond et al., 2019). Moreover, several legislative issues such as the lack of regulations across international borders and specific government standards related to the production, processing and commercialization of insects present barriers to the growth of the insect sector (Lähteenmäki-Uutela et al., 2021). The safety of *T. molitor* and *L. migratoria* insect extracts are limited to dried, freeze-dried, and ground animals by the EFSA Panel on Nutrition, Novel Foods and Food Allergens (NDA) (2021a, 2021b). Consequently, the commercialization of other processed insect products such as protein extracts, oils or chitin in the EU requires another 'specific' food authorization. In addition, the use of insect extracts for feed in the poultry, pig and aquaculture industry is not allowed. Edible insects are currently used in the food and feed sector as follows: (1) whole (dried, frozen, pre-cooked); (2) processed; and (3) extracts (EFSA Scientific Committee, 2015). The latter final products (protein, fat, and/or chitin) are probably the least explored from a technology and market perspective. These extracts are currently under investigation for their potential to increase the economic viability of the insect value chain and open new markets without compromising the environmental sustainability of the food system.

A recent study estimates that the global edible insect market value will reach USD 8 billion by 2030 (Pippinato et al., 2020); however the current forecasts do not consider the full potential of insect production. Thus, the edible insect market would strongly benefit from

the low-cost processing of extracted insect fractions compared to other insect products (whole insects, insect powder).

ii) Sustainable food production

Edible insects as a source of food and feed have gained attention both in developed and developing countries due to growing concerns over food system sustainability (Pippinato et al., 2020). The high potential as an alternative feed ingredient is related to the possibility of mass rearing insects on by-products to promote a circular economy with zero waste. These goals are reflected in the UN Sustainable Development Goal and in the European Green Deal (European Commission, 2019) and its Farm to Fork Strategy. Moreover, insect production has several benefits including 1) short reproduction cycles which translates into a quick biomass build-up; 2) efficient use of non-renewable sources such as water and soil; 3) reduced environmental footprint (Cadinu et al., 2020).

iii) Reduction of antibiotic resistance

Insects are an excellent biomass to extract different valuable antimicrobials compounds thanks to their resilience against exposure to different parasites. The molecules of their body exhibit constitutive antimicrobial activity (intrinsic), while induced metabolites counteract infections (extrinsic). The great variety of insects in nature implies that they are sources of novel molecules that could inspire the design of novel antibiotics. One of the main advantages of insect antimicrobials is that they trigger low rates of intrinsic drug resistance mechanisms which are becoming more common with the overuse of traditional antibiotics (Wang et al., 2016; Jackman et al., 2020; Li et al., 2021). Also, no horizontal transfer of bacterial resistance genes and target site mutations that lead to acquired resistance are reported (Józefiak & Engberg 2017). Therefore, a new generation of antimicrobials may be produced from insects to counteract the antimicrobial resistance era.

Conclusions and perspectives

This review illustrated the properties of insects as a valuable frontier in food and feed applications. In particular, insects have an underestimated amount of antimicrobial compounds present in their body components (intrinsic antimicrobial activity) like lauric

acid and chitin/chitosan, and intracellularly as circulating metabolites (extrinsic antimicrobial activity) in the form of AMPs.

Further investigation is required into the production of insect extracts, their specific application in food and feedstuff, and their molecular mechanism against undesired microorganisms. Research should be devoted to the development of efficient, environmentally friendly, and low-cost extraction processes. Moreover, continued in situ studies should evaluate the effectiveness of insect antimicrobials as preservatives. This may fill in the gaps needed to define more detailed legislation on the use of insect extracts as antimicrobials. Finally, understanding the mechanism of action of insect-derived compounds, the specific cellular target, and the type of microorganisms inhibited, is needed to expand the knowledge on antimicrobial properties of insects and prove the low risk of bacterial resistance development against insect antimicrobials.

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Chapter 3.1

Antimicrobial Biomasses from Lactic Acid Fermentation of Black Soldier Fly Prepupae and Related By-Products

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Abstract

Worldwide, thousands of insect species are consumed as food or are used as feed ingredients. Hermetia illucens, 'black soldier fly', is one of them, and a large amount of puparia and dead adults flies are accumulated during rearing. These materials represent important wastes but no studies are still present in the literature regarding their functional properties and potential reuse. Lactic acid bacteria (LAB) are a heterogeneous group of bacteria contributing to various industrial applications, ranging from food fermentation, chemicals production to pharmaceuticals manufacturing. A LAB feature of industrial interest is their ability to produce antimicrobial metabolites. Considering the scientific and commercial interest in discovering novel antimicrobials, this work will be direct towards fermentation of insect-derived biomasses: puparia and adults insect at the end of life cycle. To the best of our knowledge, the in vitro antimicrobial activity of fermented insects is tested for the first time. This study aimed also to evaluate differences in the composition between fermented and unfermented insects, and to study whether the fermentation and the type of LAB used played a crucial role in modifying the composition of the substrate. Results firstly highlighted fermentability of this species of insects, showed that fermented black soldier flies puparium possess a high antimicrobial activity against tested pathogens. Moreover, result of chemical composition showed that fermented biomass had a higher percentage of fat and a more complex fatty acids profile.

Introduction

The world population is predicted to increase from 5.4 billion to about 9 billion within a few decades, and it is relevant to find new sources of high-quality protein, different from soybean or animal sources, to feed this additional population (Food and Agriculture Organization of the United Nations, 2013).

To accommodate this number, insects represent a good alternative in terms of protein quality and environmental sustainability (Garofalo et al., 2019). They grow and reproduce easily, have low feed conversion rate compared to conventional livestock animals (one kg of insect biomass can be produced from 1.7 Kg of feed biomass, depending on species) and can be reared on agro-industrial by-products (Jansson & Berggren, 2015; Oonincx & de Boer, 2012). In addition to serving as feed, insects provide a high nutritional profile for humans, although the presence of allergens represents a potential hazard that must be further investigated (Leni et al., 2020; Varelas, 2019). Overall, it is demonstrated that insects production involves low energy, land area utilization and environmental footprints (Makkar et al., 2014).

This study focused on *Hermetia illucens*, also called black soldier flies (BSF), a Diptera belonging to Stratyiomidae family. In accordance with Commission Regulation (EU) 2017/893 (EU, 2017), this insect is one of the seven species approved for feeding of aquaculture animals and thanks to its high nutritional value (De Smet et al., 2018), and to its ability to convert industrial waste (Barbi et al., 2020; Bortolini et al., 2020; Gold et al., 2018; Hadj Saadoun et al., 2020; Jucker et al., 2017) is one of the most promising to be used in the feed industry.

Its life cycle includes six larval stages concluding with the prepupa stage. At this point, BSF moves away from the growth substrate and looks for a dry and safe place where it is possible to pupate (pupa stage), performing the metamorphosis and emerging as an adult (Salomone et al., 2017).

Currently, BSF larvae are reared for aquaculture in the USA (De Smet et al., 2018), for pet feed in Germany (Y.-S. Wang & Shelomi, 2017) and are included in the list of insect species with the greatest potential to be used also as food with restriction, for all of these cases, on

the substrates fed to BSF that must contain products of non-animal origin (EFSA, 2015; EU, 2017).

As a result, there has been an increase in BSF rearing at industrial-scale production in the last years (Meticulous Research, 2020). In an industrial mass rearing of BSF, besides frass, several other wastes are generated during the development of the insect. Among these, there are the puparia, which are the shells left after the adult emergence, and the bodies of the dead adults after mating and egg laying. Nowadays, these materials represent a significant waste and no studies are present in literature regarding their functional properties and potential reuse.

In the last decades, several studies reported that one possible strategy to add value to waste material are fermentation processes. Lactic acid bacteria (LAB), a heterogeneous group with recognition as Qualified Presumption of Safety (QPS)-status by EFSA, are the most important microorganisms associated with fermentation and thus exploited for industrial bioprocesses. Recently, the exploitation of LAB strains to valorize by-products and waste into high value-added products has been described but their potential on bio-transformations is still an untapped biotechnology resource (Ricci, Cirlini, et al., 2019; Ricci, Diaz, et al., 2019; Sun et al., 2015). Different metabolites of industrial interest, such as bioactive molecules, can be produced by lactic acid fermentation starting from low-cost substrates. For instance, LAB have a wide range of antimicrobial effects against many pathogens due to the production of organic acids, hydrogen peroxide, and bacteriocins and their ability to convert agro-industrial leftovers into antimicrobial compounds has been recently reported (Reis et al., 2012; Ricci, Bernini, et al., 2019). Furthermore, due to these characteristics, fermentation could also be useful to extend shelf life and improve the taste of insects (Borremans et al., 2018). The metabolic potential of LAB has been studied in different substrates and insects represent a new resource to be explored to produce high-value biochemical molecules.

In this study, we describe the fermentation of BSF and BSF-derived biomasses: prepupae, puparia and adults insects at the end of life cycle were used as substrates for LAB growth and the antimicrobial activity against different pathogens was determined by challenge test.

Differences in the chemical composition of BSF biomasses before and after fermentation, in terms of gross composition, lipid and protein profiles, were discussed. To the best of our knowledge, this is the first study focused on biovalorisation of BSF and this type of waste.

Materials and Methods

Collection of Insect Waste

The BSF prepupae, puparia and dead adult flies used for all the experiments were obtained from the Laboratory of Applied Entomology—BIOGEST-SITEIA, Department of Life Science, University of Modena and Reggio Emilia, Reggio Emilia (RE), Italy.

The BSF material was obtained from a colony initiated in 2016, from larvae purchased from CIMI Srl (Cuneo, Italy (CN)). The larvae were reared at 27 °C and 70% relative humidity and fed *at libitum* with Gainesville Housefly diet (Hogsette, 1992; Sheppard et al., 2002). The prepupae were collected and placed in a dry and ventilated polyethylene container for flies emergence. The newly emerged adults were further transferred in BugDorm® (BD4S3030, MegaView Science Co., Taiwan) provided with sugar cubes and water for adult nutrition and a patent-pending device (Benassi et al., 2018) as oviposition site for eggs laid by the BSF females. The colony maintenance was performed three times per week when the eggs were collected and placed directly on the rearing substrate for the hatching of the new larvae, whereas the puparia and dead flies were collected and kept in falcon vials at −20 °C.

Bacterial Strains

Two bacterial strains, *Lacticaseibacillus rhamnosus* 1473 isolated from Parmigiano Reggiano cheese, and *Lactiplantibacillus plantarum* 285, isolated from Brazilian cheese, were used for fermentation. The antimicrobial activity of insect waste was tested toward 3 pathogenic strains belonging to *Salmonella enterica* (serotype Rissen), *Listeria monocytogenes* (LMG 21264) and *Escherichia coli* (k88 isolated from piglet's gut). All the strains belonged to the collection of the Department of Food and Drug (University of Parma, Parma, Italy).

Lactobacilli were maintained at −80 °C in De Man, Rogosa, and Sharpe (MRS) broth (Oxoid, Basingstoke, UK) supplemented with 12.5% glycerol (*v*/*v*). Three pathogenic strains were maintained at −80 °C in tryptic soy broth (TSB) (VWR, Leuven, BE) supplemented with 12.5% glycerol (*v*/*v*).

Fermentation

Puparia and adults were smashed in small particles using IKA A10 laboratory grinder (IKA Werke GmbH e Co., Staufen, Germany), water (70% *v*/*w*) and sugar (8.5% *w*/*w*) were added, then sterilized in autoclave at 121° for 20 min in a glass jar. Prepupae were smashed in small particles using IKA A10 laboratory grinder, added of sugar (8.5% *w*/*w*) and sterilized in autoclave at 121 °C for 20 min in a glass jar. Before fermentation, LAB strains were transferred twice in MRS broth (3% *v*/*v*) and incubated for 24 h at 30 °C for *L. plantarum* 285 and 37 °C for *L. rhamnosus*1473. Afterward, MRS broth was inoculated (3% *v*/*v*) with each revitalized strain and incubated for 15 h at the specific temperatures of each species, to obtain a cell concentration of 9 Log CFU/mL. Each grown bacterial culture was centrifuged (12,857× *g*, 10 min, 4 °C), washed twice in Ringer solution (VWR, UK), and suspended in sterile bidistilled water. 30 g of puparia, adults and prepupae were inoculated individually with each bacterial suspension to obtain a final concentration of 7 Log CFU/g, in triplicate. The inoculated substrates were then incubated for 72 h at 30 °C for *L. plantarum* 285 and at 37 °C for *L. rhamnosus* 1473. For microbial counts, 5 g of each sample were homogenized in 45 mL of Ringer solution for 60 s in a Stomacher 400 Circulator (Seaward, England), and serial 10-fold dilutions were performed. Total viable count of the starter was determined using plate count agar on MRS.

Proximate Composition

Standard procedures (Association of Official Analytical Chemist, 1950) were used to test moisture, lipid and ash composition of the samples, grinded for 2 min with IKA A10 laboratory grinder before each analysis. Moisture was determined in oven at 105 °C for 24 h. An automatized Soxhlet extractor (SER 148/3 VELP SCIENTIFICA, UsmateVelate, Italy) was used to determine crude fat, extracted using diethyl ether as solvent. Total ash was determined after mineralization at 550 °C for a total time of 10 h (5 h + 5 h). Total protein content of the samples was calculated from the sum of the amounts of amino acids, determined as in paragraph 2.5. To determine the correct protein amounts, the mmoles of each amino acid were multiplied for their residual molecular mass (molecular mass of free amino acid subtracted of the molecular mass of water). Regarding total chitin content of

fermented sample, it was estimated as the sum of free glucosamine released after acid hydrolysis as described in the previous section.

Total Amino Acid Profile

Total amino acids determination was carried out as reported by the method described by Leni et al. (2019). All samples (500 mg) were hydrolyzed with 6 mL of HCl 6 N at 110 °C for 23 h. At the end of hydrolysis, 7.5 mL of 5 mM Norleucine in HCL 0.1 N, used as internal standard, was added. After filtration, the sample was brought up to volume of 250 mL. Only for the analysis of cysteine, determined as cysteic acid, the acid hydrolysis described above was preceded by a performic acid oxidation. In this case, 2 mL of performic acid freshly prepared (by mixing formic acid with hydrogen peroxide in 9:1 proportion) was added to an amount of 0.5 g sample and kept in an ice bath for 16 h at 0 °C. Then the bromine formed after the addition of 0.3 mL of hydrobromidric acid was removed under nitrogen flow. The hydrolyzed samples were analyzed by UPLC/ESI-MS, after derivatization with reconstituted AccQ Tag reagent (Waters Co., Milford, USA) according to the method described by Leni et al. (2020). Calibration was performed with standard solution prepared mixing 133.3 μL of Norleucine (5 mM), 133.3 μL of amino acids hydrolysate standard mixture (2.5 mM), 133.3 μL of cysteic acid in HCL 0.1 N (2.5 mM) and 100 μL of deionized water.

Tryptophan Determination by UPLC/ESI-MS after Alkaline Hydrolysis

Tryptophan analysis was carried out according to the method described by Caligiani et al. (2018) with some modifications. 3 mL of 4 N NaOH and 150 μL of 5-methyl- DL-tryptophan standard solution (prepared by mixing 16 milligrams in 100 mL of distilled water) were added to 0.2 hundred milligrams of sample and hydrolyzed at 110 °C for 18 h. After alkaline hydrolysis, the solution was neutralized by adding 37% HCl and brought to 25 mL with sodium borate buffer (0.1 M, pH 9.0). The samples were centrifuged for 5 min at 4000 rpm at 4 °C. 0.45 μm nylon filter membrane was used to filter the surnatants collected after centrifugation. UPLC/ESI-MS analysis was performed by using an ACQUITY UPLC separation system with an Acquity BEH C18 column (1.7 μm, 2.1 × 150 mm). The mobile phase was composed by H2O + 0.2% CH3CN + 0.1% HCOOH (eluent A) and CH3CN + 0.1%

HCOOH (eluent B). Gradient elution was performed: isocratic 100% A for 1.8 min, from 100% A to 50% A by linear gradient in 11.4 min and 0.8 min at 50% A plus washing step at 0% A (100% B) and reconditioning. Flow rate was set at 0.25 mL/min, injection volume 2 μL, column temperature 35 °C and sample temperature 23 °C. Detection was performed by using Waters SQ mass spectrometer: ESI source in positive ionization mode, capillary voltage 3.2 kV, cone voltage 30 V, source temperature 150 °C, desolvation temperature 300 °C, cone gas flow (N2): 100 L/h, desolvation gas flow (N2): 650 L/h, full scan acquisition (100–2000 m/z), scan duration 1 s.

Determination of Chitin

The total amount of chitin was determined by using a UPLC/ESI-MS quantification of glucosamine after samples acidic hydrolysis, as described by D'Hondt et al. (2020) with some modifications. Practically, samples preparation was the same used for total amino acids determination: 500 mg of BSF puparia, dead adults and prepupae were hydrolyzed in 6 N HCl for 23 h at 110 °C, added to 7.5 mL of 5 mM Norleucine as internal standard, filtered and brought to 250 mL. The same procedure was applied to a chitin standard (Sigma Aldrich) to calculate the recovery. UPLC/ESI-MS Analysis conditions were the same described for tryptophan determination.

Determination of Fatty Acids Profile by GC-MS

The determination of fatty acids profile was carried out on the crude fat extracted using the Soxhlet extraction technique. Before the analysis in GC-MS, acidic-catalyzed transmethylations were carried out on 50 mg of BSF fat residue according to the method used by Caligiani et al. (2018) with some modifications. Weighed fat of each BSF sample was dissolved in 1 mL of 5% HCl in methanol. The reaction was carried out in oven at 70 °C for 45 min. After cooling, 50 μL of methyl tetracosanoate, used as internal standard, and 2.5 mL of hexane were added. The superior hexane phase containing the fatty acid methyl esters formed during the acid transmethylation was collected and stored. Before the instrumental analysis, a dilution of each extract was performed by adding differents quantities of hexane to match the linearity range of the GC-MS instrument. The solutions were split injected (1 μL) on a Thermo Scientific Trace 1300 gas-chromatograph (Thermo Scientific, Waltham,

MA, USA) carrying a Supelcowax ms capillary column (30 m, i.d 25 mm, Supelco, Bellafonte, USA) coupled to a Thermo Scientific Trace ISQ mass spectrometer (Thermo Scientific, Waltham, MA, USA). Carrier gas was helium (1 mL/min), injector and detector temperatures were kept at 250 °C, while oven temperature was programmed from 80 to 240 °C at 20 °C/min. Content of each single fatty acid was calculated in relation to the concentration of the internal standard, after calculating the response factors using the Supelco® 37 Component FAME Mix (Sigma Aldrich, Saint Louis, MO, USA). Finally, results were expressed as relative percentage of each fatty acid.

Microbial Challenge Test

Challenge tests were carried out to evaluate the growth potential of *Listeria monocytogenes* LMG 21264, *Escherichia coli* K88, *Salmonella* RISSEN in fermented and unfermented samples, in triplicate. Before use, pathogenic strains were cultured twice, for 24 h at 37 °C, with a 3% *v*/*v* inoculum in TSB added with 0.6% yeast extract (Oxoid, Basingstoke, UK). Afterward, TSB broth was inoculated (3% *v*/*v*) with each revitalized strain and incubated for 15 h at 37 °C, to obtain a cell concentration of 9 Log CFU/mL. Each bacterial culture was then centrifuged (12,857× *g*, 10 min, 4 °C), washed twice in Ringer solution, and suspended in sterile bidistilled water. Puparia, adults, prepupae fermented with both strains and unfermented (control), were inoculated, individually, with each bacterial suspension of pathogen, to obtain a final concentration of 6–7 Log CFU/g. The inoculated substrates were incubated at 37 °C and analyzed immediately after the inoculum (T_0), after 24 h (T_{24}) and after 48 h (T_{48}) of incubation. For microbial counts, 5 g of each sample were homogenized in 45 mL of Ringer solution for 60 s in a Stomacher, and serial 10-fold dilutions were performed. Total viable count was determined using plate count agar on selective medium for each pathogen: Microbiology Chromocult (Merck KGaA, Darmastadt, DE) for the detection of *Salmonella* Rissen and *Escherichia coli* and Listeria selective agar base acc. Ottaviani and Agosti (ALOA) (VWR, Leuven, BE) for *Listeria monocytogenes*.

Statistical Analysis

Data were statistically elaborated by SPSS Statistic 23.0 software (SPSS Inc., Chicago, IL, USA). One-way ANOVA was applied for analyzing the results of microbial challenge test,

post hoc test Tukey's HSD was applied to evaluate significant difference ($p < 0.05$) among the means of different samples at different times. Pearson's correlation between growth ability during fermentation and decrease of pathogenic strains in microbial challenge test was analyzed.

Results

Fermentation

The fermentation of insect waste (puparia and adults insects) and prepupae was carried out by inoculating two different LAB strains, *L. plantarum* (285) and *L. rhamnosus* (1473), at the concentration of 7 Log CFU/g. After 72 h of incubation at the optimal temperature for each species (30 °C for *L. plantarum* and 37 °C *L. rhamnosus*), the strains showed different growth abilities (Table 1). *L. plantarum* was able to grow in puparia and adults with an average of 2 Log CFU/g while in prepupae there was an initial decrease from the original inoculum of about 2 Log CFU/g (T_0). Conversely, *L. rhamnosus* were able to grow only in puparia, with an increase of ca. 2 Log CFU/g, while in prepupae a decrease of about 5 Log CFU/g from initial inoculum was recorded. In adults, although a reduction occurred after inoculum, the growth of *L. rhamnosus* was restored up to 3 Log CFU/g at 72 h.

Table 1. Bacterial counts of two different lactic acid bacteria (LAB) strains in prepupae, puparia and adults of BSF after initial inoculum and after 72 h of fermentation. Data are reported as Log CFU/g (average values ± standard deviation).

	***L. plantarum* 285**			***L. rhamnosus* 1473**		
	T_0	**T_{72}**	**Δ (T_{72}–T_0)**	**T_0**	**T_{72}**	**Δ (T_{72}–T_0)**
Prepupae	5.61 ± 0.68	7.80 ± 0.57	2.19	4.81 ± 1.30	2.64 ± 0.36	−2.17
Puparia	7.19 ± 0.28	9.36 ± 0.10	2.17	7.15 ± 0.64	9.11 ± 0.29	1.96
Dead Adults	6.65 ± 0.43	8.84 ± 0.48	2.19	4.29 ± 0.81	7.33 ± 0.49	3.04

Black Soldier Fly Composition

In order to investigate the characteristics of the different fermented samples as a function of the different LAB strains used for fermentation, a complete chemical composition analysis of the biomasses obtained from fermentation of BSF prepupae, puparia and dead adults were carried out. The composition in moisture, protein, crude fat, chitin and ash, expressed on wet mass is reported in Table 2.

Table 2. Proximate composition of fermented BSF dead adults, puparia and prepupae with two different LAB strains. * Values are expressed on wet matter basis and are the result of four replicate analyses. To be considered for each sample a Sugar content equal to 8.5% added for fermentation process.

Composition (%) *	***L. plantarum 285***			***L. rhamnosus 1473***		
	Prepupae	**Puparia**	**Dead Adults**	**Prepupae**	**Puparia**	**Dead Adults**
Moisture (Oven, 105 °C 24 h)	67 ± 0.3	68.4 ± 2.00	76.4 ± 0.40	68.07 ± 0.02	74.0 ± 1.70	76.51 ± 0.05
Lipid (Soxhlet)	5.3 ± 0.5	1.7 ± 0.60	3.85 ± 0.01	3.30 ± 0.90	2.28 ± 0.03	4.30 ± 0.10
Proteins, from total AA (UPLC/ESI-MS)	11 ± 1.00	7 ± 1.00	9.34 ± 0.00	11.15 ± 0.06	6.4 ± 0.30	9.60 ± 0.70
Chitin (UPLC/ESI-MS Determination of Glucosamine)	1.8 ± 0.20	5.2 ± 0.60	1.29 ± 0.09	1.70 ± 0.10	3.9 ± 0.30	1.4 ± 0.10
Ash (Oven 550 °C 5 h + 5 h)	2.07 ± 0.06	5.75 ± 0.02	1.00 ± 0.10	2.00 ± 0.10	4.7 ± 1.10	0.82 ± 0.07

BSF dead adults and prepupae resulted to be higher in fat respect to puparia. This is explained by the fact that puparia is the protective shell of insects, while the fat accumulation takes place largely inside the insect's body. Lower amount of lipids are found in BSF prepupae fermented by *L. rhamnosus* respect to the corresponding sample fermented by *L. plantarum.*

Protein content of fermented dead adults and prepupae is higher compared to puparia, in agreement with the data present in the literature (X. Liu et al., 2017). The LAB strain utilized for fermentation does not affect the protein content.

The chitin content, as expected, is higher in fermented puparia samples. Puparia fermented with *L. rhamnosus* and *L. plantarum* respectively reached the levels of 3.9% and 5.2%. In parallel with chitin content, ashes reached the highest level in fermented puparia.

As a whole, the gross composition of the fermented biomasses showed that the main differences can be found in the different insect materials used as substrates of fermentation,

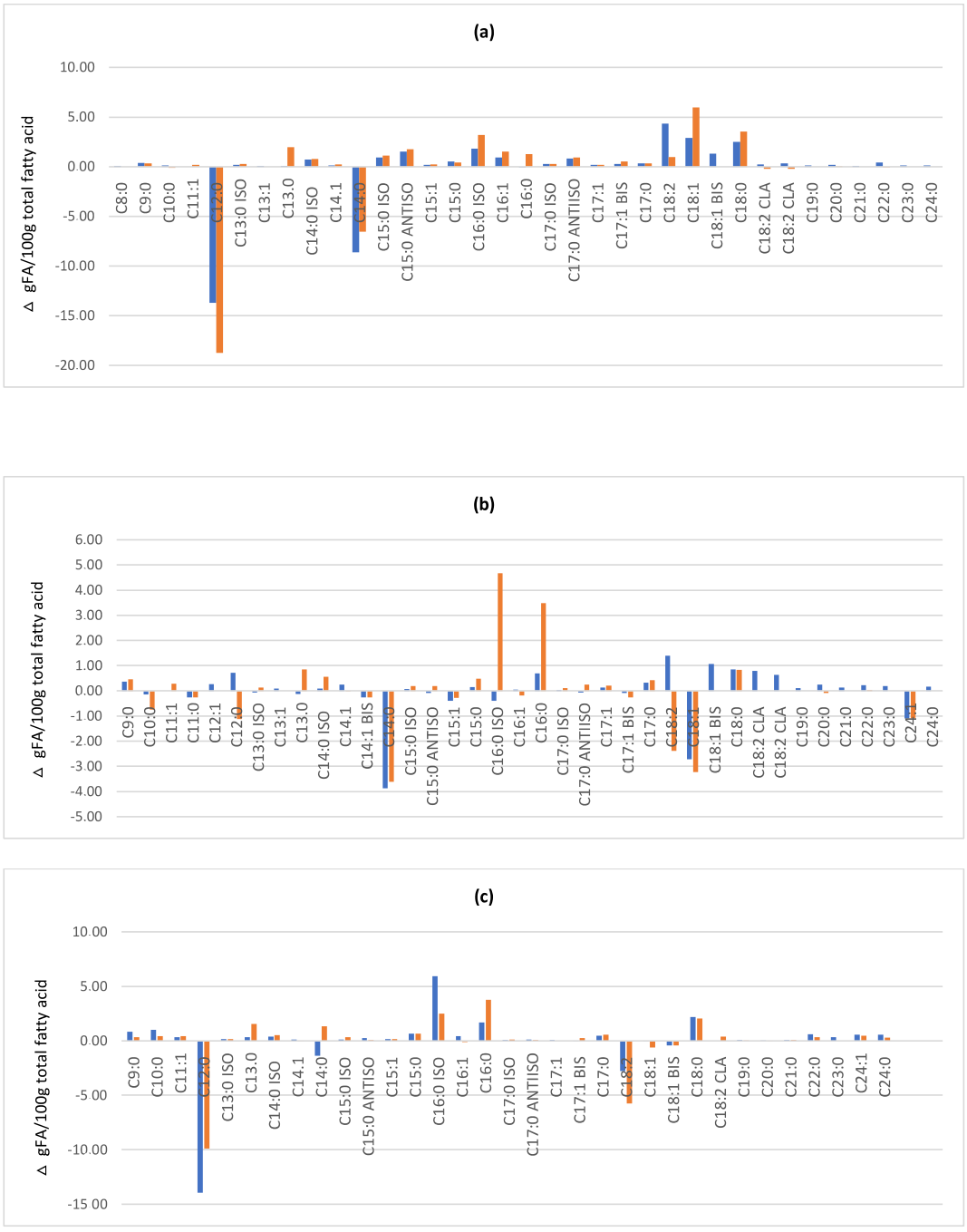
while only slight effects can be attributed to the different LAB strains used. To better understand at a molecular level the composition of fermented samples and to evaluate possible differences between fermented and unfermented samples, fatty acid profile and total amino acid profile were further analyzed and compared to the unfermented insect biomasses used as starting material for fermentation.

Variations of Fatty Acid Profile after Fermentation

Fatty acid profile varied considerably after fermentation. The differences between fatty acids distribution in BSF before and after fermentation is shown in Figure 1, with the graph base line representing the starting composition of the unfermented sample, and the bars indicating how much lipid composition changed after fermentation with two different strains. Full data on fatty acid composition is reported in Table S1 of the Supplementary Material. The data showed a consistent fatty acids redistribution. In most cases, there was a reduction of the typical fatty acid of black soldier flies, such as Lauric acid (C12:0) and an increase of minor fatty acids as short-chain fatty acids, odd-chain fatty acids, branched-chain fatty acids, typical of the bacterial cell wall (Zhang & Rock, 2008).

Therefore, the lipid biomass shifted from a typical composition of insects to a composition containing also fatty acids from lactic acid bacteria metabolism, showing that, in general, the fermentation process modified the insect biomass lipid fraction.

Figure 1. Variations of fatty acids distribution, expressed as relative percentage, in fermented by *L. rhamnosus* (first line)/fermented by *L. plantarum* (second line) BSF prepupae (**a**), puparia (**b**) and dead adults (**c**) compared to corresponding unfermented sample (graph base line).

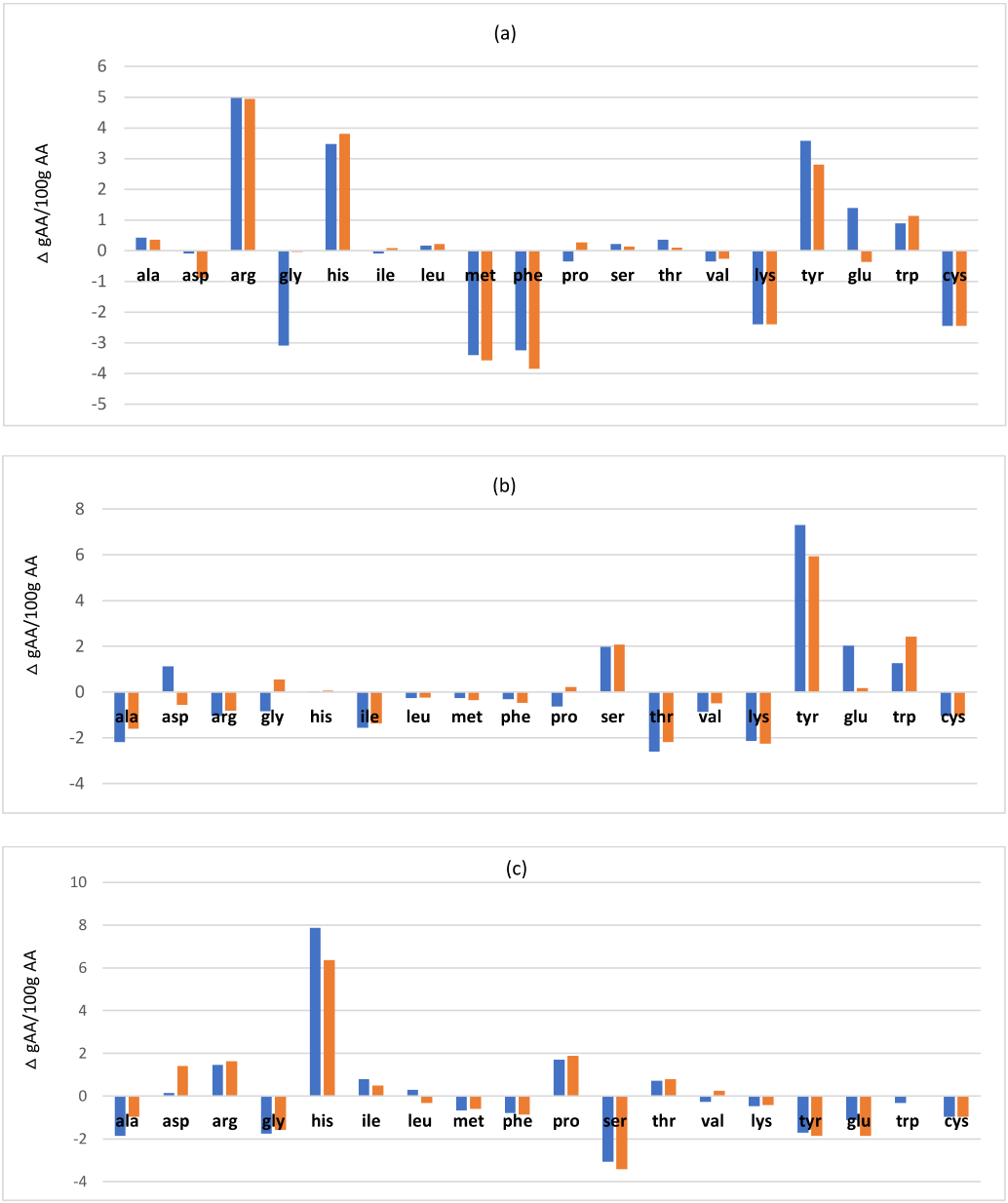


Variations of Total Amino Acid Profile after Fermentation

In order to deeply investigate the effect of fermentation on protein fraction, total amino acids profile of the fermented BSF samples was assessed and compared with unfermented samples. Complete data are reported in Table S2 of the Supplementary Material. Figure 2 displays the variation in the total amino acid distribution between BSF fermented and

unfermented, expressed in gAA/100 g AA. The graph baseline represents the unfermented sample and the bars indicated how total amino acid composition changes for the fermented ones.

Figure 2. Variations of total Amino Acid distribution, expressed as relative percentage, in fermented by *L. rhamnosus* (first line)/fermented by *L. plantarum* (second line) BSF prepupae (**a**), puparia (**b**) and dead adults (**c**) compared to corresponding unfermented sample (graph base line).



The detailed composition of the total amino acids revealed that there was, actually, an amino acids redistribution after fermentation, also depending on the LAB strain, despite the total amount of protein was similar for the two LAB strain used (see Table 2), suggesting that *L. rhamnosus* and *L. plantarum* presented a different protein metabolism.

For the fermented samples of dead adults, the effect of the two LAB strains was very similar, and the most relevant differences before and after fermentation were in the enhanced amount of histidine, proline, arginine, and in the reduction of alanine, glycine, serine, glutamic acid and tyrosine.

In the case of puparia, different changes, common to both LAB strains, were observed: a reduction of arginine, alanine, isoleucine, lysine, cysteine, threonine. Serine, tyrosine and tryptophan were instead in higher amount respect to unfermented puparia sample. A different behavior of the two strains was instead observed regarding aspartic and glutamic acid, glycine and proline, both in puparia and prepupae samples. In the samples of prepupae, it was observed a higher amount of arginine, tyrosine, serine and tryptophan after fermentation, and a reduction of methionine, phenylalanine, cysteine and lysine

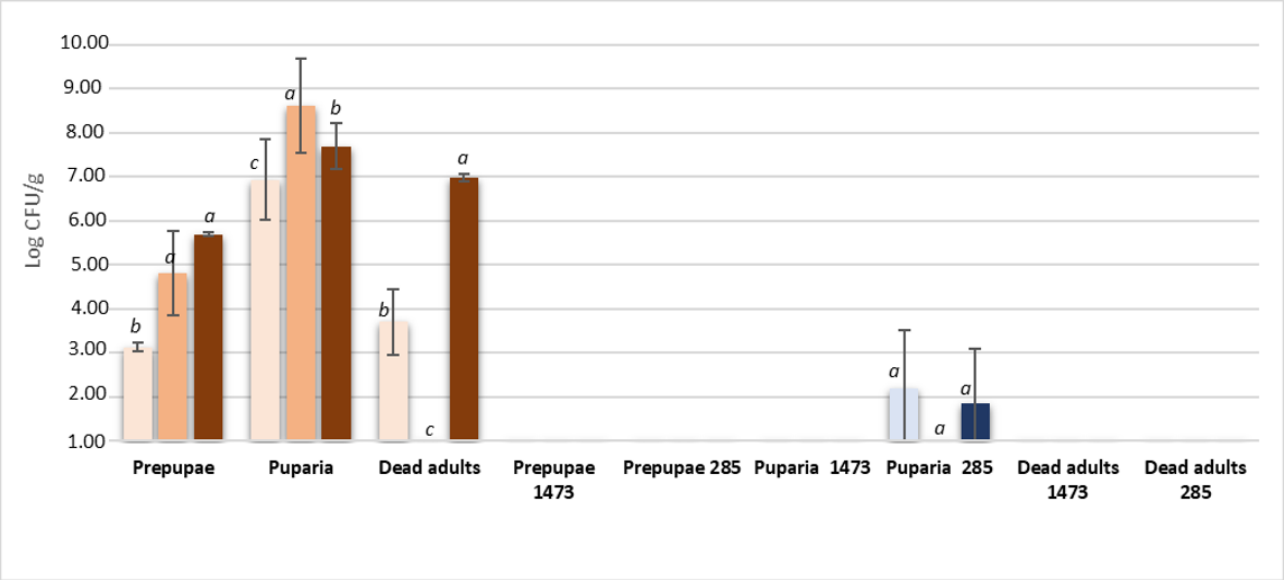
As a whole, these results suggest that LAB strains used were able to modify the protein composition of the biomass to a different extent, depending on both the specific LAB strain and the insect biomass.

Antimicrobial Activity

The in vitro antimicrobial activity was carried out by microbial challenge tests, using three pathogenic strains belonging to *L. monocytogenes*, *Salmonella* spp., *E. coli*. The antimicrobial activities of the insect waste (puparia and bodies of dead adult insects) and prepupae were determined by evaluating the growth of different pathogens in fermented and unfermented samples on selective medium for each pathogen.

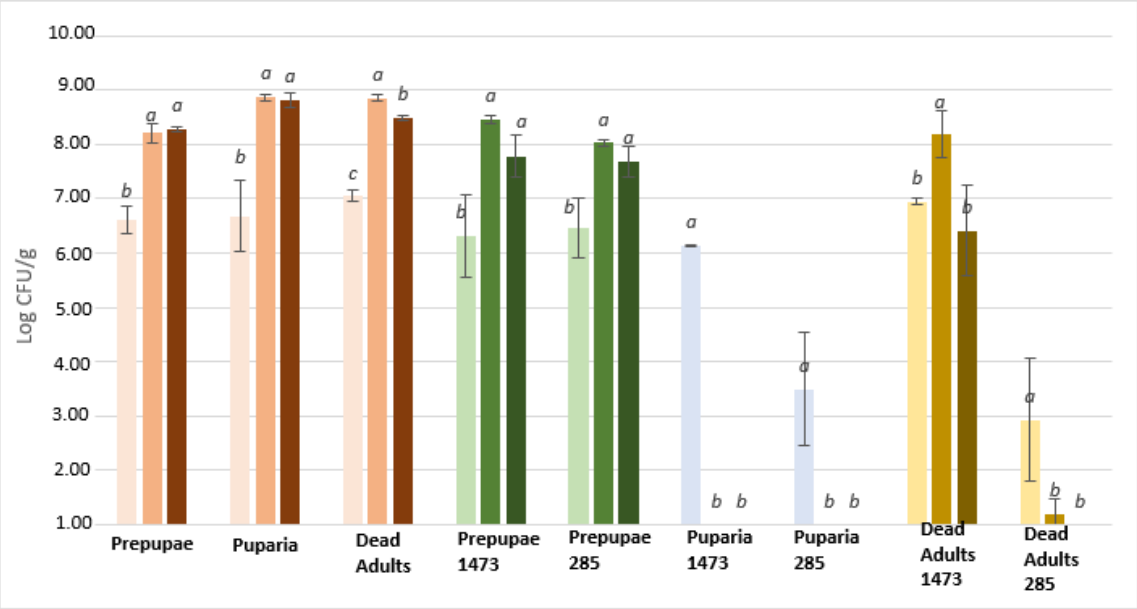
Unfermented samples inoculated with *Listeria monocytogenes* LMG 21264 (Figure 3) highlight a significant microbial count reduction of about 3 Log CFU/g ($p < 0.05$) after original inocula (ca. 6 Log CFU/g) in prepupae and adults. This trend is not maintained during incubation because in prepupae there was an increase in concentration after 24 h and 48 h, while in adult samples, after an initial decrease there was an increase of 4 Log CFU/g at 48 h. In puparia growth of *Listeria monocytogenes* is recorded after inoculum and after 24 h, while there is a slight decrease after 48 h (ca. 0.90 Log CFU/g) probably due to loss of viability. Moving to all fermented samples, a rapid reduction in total viable cells under the detection limit (1 Log CFU/g) was reached after inoculum (T_0) and maintained for 48 h of incubation. Interestingly, no correlation was detected between the ability of the strains to grow in insect waste during fermentation and the antimicrobial activity.

Figure 3. Growth of *Listeria monocytogenes* LMG 21264 on fermented/unfermented insect waste after inoculum (first line/light colour), 24 h (second line/medium colour), and 48 h (third line/dark colour). Starting inoculum 6 Log CFU/g. Letters a-c mark significant ($p < 0.05$) differences among the samples. 1473: fermented with *L. rhamnosus*; 285: fermented with *L. plantarum*.



Regarding microbial challenge test with *Salmonella* Rissen (Figure 4) a different trend was observed. In this case, unfermented samples didn't show a decrease in pathogen load. The concentration after inoculum is nearly about the original inocula, ca. 7 Log CFU/g, and an increase of about 1 Log CFU/g was recorded after 24 h.

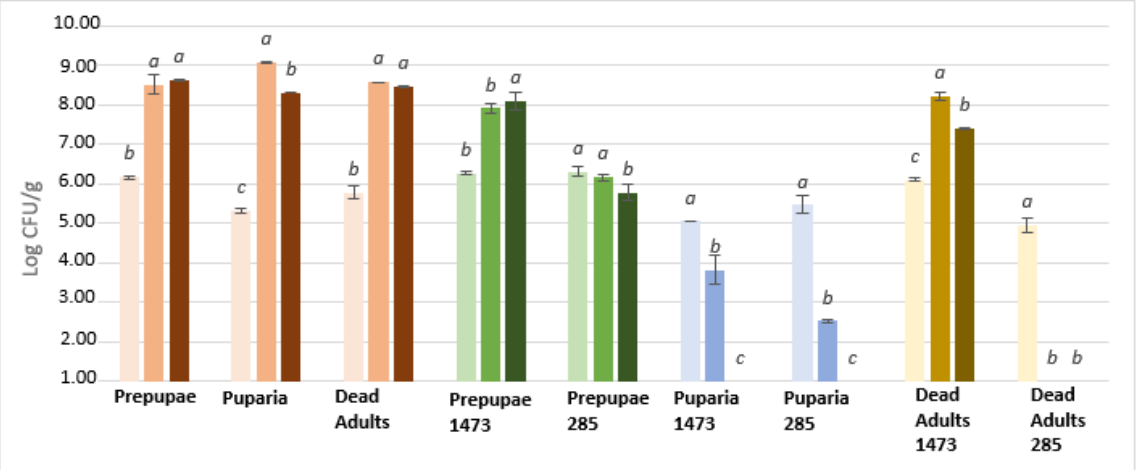
Figure 4. Growth of *Salmonella* Rissen on fermented/unfermented insect waste after inoculum (first line/light colour), 24 h (second line/medium colour), and 48 h (third line/dark colour). Starting inoculum 7 Log CFU/g. Letters a-c mark significant ($p < 0.05$) differences among the samples. 1473: fermented with *L. rhamnosus*; 285: fermented with *L. plantarum.*



Differently from the microbial challenge test with *Listeria monocytogenes*, only three of fermented samples show an antimicrobial activity: puparia fermented with both LAB strains and adults fermented with *L. plantarum*. In particular, a significant reduction ($p < 0.05$) of concentration of pathogens is recorded after inoculum, but a further drop was shown after 24 h until 48 h. On the other hand, fermented prepupae and adults fermented with *L. rhamnosus* revealed similar behavior to unfermented insects. For these samples a correlation ($r = 0.69$; $p < 0.05$) between microbial counts and antimicrobial activities was detected.

Finally, considering *Escherichia coli* K88, a decrease of its microbial count was observed at T_0 in all unfermented substrates, but after 24 h and 48 h, the growth was restored (Figure 5). Overall, in fermented prepupae and adults fermented with *L. rhamnosus* it was not observed a relevant antimicrobial activity while for the other fermented samples (Puparia 1473, Puparia 285, Dead Adults 285) a trend similar to *Salmonella* was shown.

Figure 5. Growth of *Escherichia coli* k88 on fermented/unfermented insect waste after inoculum (first line/light colour), 24 h (second line/medium colour), and 48 h (third line/dark colour). Starting inoculum 7 Log CFU/g. Letters a-c mark significant ($p < 0.05$) differences among the samples. 1473: fermented with *L. rhamnosus*; 285: fermented with *L. plantarum*.



Discussion

In the last decades, attention is focused to find protein sources alternative to soybean in the formulation of feed diets. This trend is justified given the fact the world population is predicted to increase and consequently also the food demand. At the same time, the increasing occurrence of outbreaks in livestock caused by pathogenic microorganisms poses the need to discover antimicrobials from natural sources. As fermentation is known to be a strategy to produce metabolites with antimicrobial activity, the rationale of this work was to explore the ability of LAB to grow on insects and their wastes looking for possible valorization of these materials in terms of antimicrobial activity. Literature reported that fermentation process is applied during the edible insect production mainly to (i) increase the digestibility and stability of waste before the use of insects; (ii) convert food and by-product wastes into ingredients of artificial diets for insects (iii) prolong the shelf-life of edible insects (Borremans et al., 2018; Varelas, 2019). Here we report the first study on the potential reuse also of insect waste, focusing on BSF, using fermentation to enhance the antimicrobials properties of raw materials. To reach this purpose, strains of *L. plantarum* and *L. rhamnosus* were used as starter for fermentation of insect waste (puparia and bodies of dead adults) and prepupae. The choice of using these strains is due to the fact that they had recently shown the ability to produce antimicrobial compounds from fermentation of vegetable by-products (Ricci, Bernini, et al., 2019). Our data demonstrated two main trends: (i) bacterial growth was observed after LAB inoculum in puparia (ii) a decrease of bacterial count after inoculum was detected and, after 72 h of incubation, cell growth was restored or

a further cell decrease was reached. Thus, interestingly we observed an intrinsic antimicrobial activity of substrates prepupae and dead adults against LAB.

The antimicrobial activity of BSF larvae reared on contaminated substrates or their extracts has been reported in other studies (Choi et al., 2012; Choi & Jiang, 2014; Q. Liu et al., 2008; Park et al., 2014, 2015). As different authors suggest, the ability of insects to live in extremely harsh environments is favored by generation of antimicrobial peptides and other substances produced on the surface or within their digestive tract to prevent microbial infection (Callegari et al., 2020).

The key strength of this work is to explore if antimicrobial activity is present at different stage of the development of BSF and if fermentation can increase it.

Interestingly, after lactic acid fermentation, the antimicrobial activity significantly increased.

To note, a strong correlation between the ability to grow and the reduction of pathogens was recorded. Indeed, puparia fermented with *L. plantarum* and *L. rhamnosus* and adults fermented with *L. plantarum* showed the highest LAB growth during fermentation and the highest antimicrobial activity against *Salmonella* Rissen and *Escherichia coli* k88. On the other hand, this trend is not recorded in microbial challenge tests with *Listeria monocytogenes* where all samples showed antimicrobial activity.

The differences in composition among the three substrates tested could explain the different trends recorded during microbial challenge test. Considering that the main components of insects are protein, fat and chitin, we can presume that during fermentation these substrates were hydrolyzed thanks to the enzymatic portfolio of the strains, generating secondary metabolites able to exert the antimicrobial action (Leroy & De Vuyst, 2004). The results obtained from molecular analysis actually confirm that insect macromolecules, especially lipids and proteins, have been affected by LAB metabolism. From the bulk composition, fermented adults and prepupae resulted to have highest protein and lipid content, while puparia had the highest chitin content. Regarding protein and lipid content, their amounts are lower than those reported in literature for unfermented samples (Caligiani et al., 2018; X. Liu et al., 2017), suggesting that lipid and protein fractions have been consumed/modified

by LAB metabolism. Analyzing more in detail both these fractions, a significant redistribution of fatty acid and amino acid profiles was indeed observed. It is known that BSF has a peculiar fatty acid profile (Ramos-Bueno et al., 2016), being lauric acid (C12:0) the most represented fatty acid in BSF prepupa and adult, whose value can be more than 50% of total detected fatty acids, followed by myristic acid (C14:0) and palmitic acid (C16:0) (Caligiani et al., 2018; Danieli et al., 2019). According to these data, a very similar fatty acid profile was observed in this work in unfermented BSF prepupae and dead adults samples. After fermentation, fatty acid composition of BSF samples was remodeled, moving to a 'mixed' profile containing both insects and LAB fatty acids, including minor fatty acids, as branched-chain fatty acids, odd chain fatty acids, short-chain fatty acids (Kaneda, 1991; Ortega-Anaya & Hernández-Santoyo, 2016). This is in agreement with other studies which showed that fermentation process could significantly change the biomass molecular composition, including fatty acid profile (Bao et al., 2016; Hao et al., 2020; D. H. Wang et al., 2019). The shift in fatty acids profile is common to all the fermented samples, therefore, despite some minor LAB produced fatty acids can have slight antimicrobial effects, the lipidic fraction alone is not able to explain the different behavior of fermented samples in the challenge tests.

Total amino acid profile of unfermented BSF samples was also remodeled after fermentation, however it is difficult to find a *rationale* behind the modifications observed, because there is a combined effect of substrate/LAB strain, generating differences among all fermented samples. This is in agreement with other studies showing the ability of LAB to modify the amino acid profile of the fermented biomasses, with differences related to microorganisms and different growth substrates used (Kieronczyk et al., 2001; J. Z. Liu et al., 2003; Tammam et al., 2000; Williams et al., 2001).

In addition, the protein-rich sample of dead adults fermented with *L. plantarum*, having the highest antimicrobial activity, showed the same variation in amino acid profile of the adults sample fermented with the other LAB strain with low antimicrobial activity, suggesting that probably the modification of the protein fraction is not the most important factor involved in the antimicrobial effect. However, among the nitrogen fraction, the presence of

antimicrobial peptides remains to be explored, eventually produced by the specific LAB strains.

To note, both strains, *L. plantarum* 285 and *L. rhamnosus* 1473, showed the best growth performance and the highest antimicrobial activity when they were grown on puparia. This insect biomass is represented only by the cuticle that covers insect body and it consists of chitin in different forms respect to the prepupae and adults because there is higher sclerotization of the cuticle proteins in the last larval stage. During sclerotization, the insect's cuticle becomes harder and protects the insect during the development and metamorphosis (Andersen, 2012). In fact, as demonstrated by gross composition, puparia contains the highest amount of chitin and minerals and the lower amount of protein and lipid, and this specificity seems to favor LAB growth and the development of antimicrobial properties against pathogens. Although proteolysis, amino acids catabolism and lipolysis are the most studied pathways, LAB have also shown the ability to grow on chitin substrates and hydrolyze it in different derivatives such as chitooligosaccharides and chitosan (Philibert et al., 2017; Rosmawati et al., 2019). Despite no specific analysis for the detailed determination of chitin or chitosan oligomers was performed, it is possible that fermentation leads to a modification of chitin with production of antimicrobial compounds. In fact, recently, antibacterial activity of chitosan and its derivatives (Haghighi, Leugoue, et al., 2020; Haghighi, Licciardello, et al., 2020; Khattak et al., 2019) has received considerable attention. These molecules have a cationic nature that could interact with negatively charged lipids, proteins, and carbohydrates present on the surface of bacterial cell, inhibiting the transport of solutes and therefore the viability of the pathogens.

The antibacterial effect of chitosan is greater than chitin and depends on the molecular weight and on its deacetylation degree. A preliminary study on genome sequence of the strains used in this work showed the presence of different chitinases (data not shown). This is consistent with other studies arguing that LAB can express the chitinolytic systems colonization factor (Leisner et al., 2008; Sánchez et al., 2011).

Conclusions

This work aimed to answer questions whether LAB can grow and ferment fractions obtained during rearing of the black soldier fly and whether it is possible to produce high added value molecules during fermentation. As a whole, puparia and adults are fermentable and after fermentation they show antimicrobial activity. The preliminary analysis on LAB fermented insect biomasses showed a shift in lipid and protein composition induced by LAB, suggesting that the fermentation caused important changes in the molecular composition of the biomass analyzed. Nevertheless, further studies are necessary to deepen the knowledge about the molecular components responsible for antimicrobial activity and to highlight the mechanism of action.

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Supplementary materials

Table S1. Total Fatty acids (FA) compositions of unfermented and fermented BSF dead adults, puparia and prepupae.*All the results are calculated as g FA/100g FA

FA* (g/100g total fatty acids)	Unf D.A.	D.A. 1473	D.A. 285	Unf Pup.	Pup. 1473	Pup. 285	Unf Pre.	Pre. 1473	Pre. 285
C8:0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.10	0.00
C9:0	0.02	0.86	0.35	0.00	0.37	0.46	0.11	0.48	0.43
C10:0	0.87	1.90	1.32	1.82	1.68	1.08	2.48	2.61	2.37
C11:1	0.00	0.33	0.42	0.00	0.00	0.28	0.00	0.00	0.21
C11:0	0.00	0.11	0.00	0.26	0.00	0.00	0.00	0.00	0.00
C12:0 ISO	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.00
C12:1	0.00	0.00	0.00	0.00	0.27	0.00	0.00	0.00	0.00
C12:0	44.05	30.11	34.17	37.24	37.96	36.11	47.09	33.37	28.36
C13:0 ISO	0.11	0.25	0.27	0.22	0.15	0.35	0.00	0.20	0.29
C13:1	0.00	0.00	0.00	0.00	0.09	0.00	0.00	0.07	0.00
C13:0	0.11	0.46	1.68	0.23	0.11	1.08	0.00	0.07	1.96
C14:0 ISO	0.33	0.70	0.85	0.36	0.44	0.91	0.00	0.71	0.79
C14.1	0.24	0.35	0.21	0.20	0.46	0.18	0.16	0.29	0.38
C14:1 BIS	0.00	0.00	0.00	0.26	0.00	0.00	0.00	0.00	0.00
C14:0	12.07	10.69	13.40	14.13	10.26	10.52	15.84	7.25	9.29
C15:0 ISO	0.31	0.44	0.67	0.28	0.36	0.46	0.00	0.94	1.12
C15:0 ANTISO	0.39	0.63	0.44	0.52	0.45	0.71	0.00	1.54	1.77
C15:1	0.07	0.24	0.23	0.52	0.13	0.24	0.00	0.17	0.24
C15:0	0.16	0.80	0.82	0.42	0.57	0.90	0.05	0.60	0.48
C16:0 ISO	0.58	6.52	3.07	1.19	0.81	5.87	0.00	1.84	3.20
C16:1	2.44	2.87	2.32	2.84	2.89	2.67	2.39	3.32	3.92
C16:0	11.50	13.20	15.28	13.71	14.41	17.19	14.26	14.19	15.53
C17:0 ISO	0.10	0.17	0.22	0.12	0.14	0.23	0.00	0.30	0.30
C17:0 ANTIISO	0.14	0.25	0.22	0.26	0.19	0.50	0.00	0.85	0.94
C17:1	0.11	0.18	0.11	0.13	0.27	0.33	0.00	0.19	0.17
C17:1 BIS	0.29	0.29	0.54	0.27	0.18	0.00	0.00	0.29	0.51
C17:0	0.15	0.63	0.71	0.32	0.65	0.75	0.08	0.44	0.44
C18:2	12.09	9.33	6.33	8.05	9.45	5.66	8.93	13.27	9.92
C18:1	10.37	10.34	9.77	11.72	9.00	8.49	6.79	9.71	12.75
C18:1 BIS	0.42	0.00	0.00	0.00	1.06	0.00	0.00	1.32	0.00
C18:0	2.19	4.36	4.26	3.60	4.44	4.43	0.96	3.48	4.52
C18:2 CLA	0.00	0.00	0.40	0.00	0.79	0.00	0.20	0.43	0.00

C18:2 CLA	0.00	0.00	0.00	0.00	0.63	0.00	0.20	0.54	0.00
C19:0	0.01	0.08	0.05	0.00	0.11	0.00	0.00	0.13	0.00
C20:0	0.12	0.13	0.09	0.09	0.32	0.00	0.01	0.18	0.03
C21:0	0.00	0.09	0.07	0.00	0.13	0.00	0.00	0.09	0.00
C22:0	0.09	0.70	0.43	0.35	0.57	0.38	0.01	0.43	0.00
C23:0	0.00	0.32	0.00	0.00	0.20	0.00	0.00	0.15	0.00
C24:1	0.00	0.57	0.47	1.10	0.00	0.00	0.00	0.00	0.00
C24:0	0.08	0.66	0.39	0.00	0.17	0.00	0.00	0.13	0.00

Unf. D.A. unfermented dead adults; D.A.1473 dead adults fermented with *L. rhamnosus* 1473; D.A. 285 dead adults fermented with *L. plantarum* 285; Unf. Pup. unfermented puparia; Pup.1473 puparia fermented with *L. rhamnosus* 1473; Pup. 285 puparia fermented with *L. plantarum* 285; Unf. Pre. unfermented prepupae; Pre.1473 prepupae fermented with *L. rhamnosus* 1473; Pre. 285 prepupae fermented with *L. plantarum* 285

Table S2. Total Amino Acid (AA) composition of unfermented and fermented BSF dead adults, puparia and prepupae. *All the results are calculated by dry mass as gAA/100g AA and expressed as means ± standard deviation of two replicate analysis.

AA (g/100g aa)*	Unf. D.A.	D.A. 1473	D.A. 285	Unf. Pup.	Pup. 1473	Pup. 285	Unf. Pre.	Pre. 1473	Pre. 285
ala	9.7±0.9	7.8±0.0	8.7±0.3	11.7±0.5	9.5±0.2	10.1±0.6	5.5±0.3	5.9±0.1	5.9±0.1
asp	10.2±1.1	10.3±1.7	11.6±0.0	7.9±0.5	9.0±0.2	7.3±0.4	8.2±0.4	8.1±0.8	7.3±1.0
arg	6.7±0.8	8.2±0.5	8.3±0.5	3.7±0.4	2.7±0.1	2.9±0.0	4.8±0.0	9.8±0.1	9.8±1.1
gly	6.8±0.4	5.0±0.0	5.2±0.0	13.8±0.7	12.9±0.2	14±1	6.0±0.0	2.9±3.5	5.9±0.1
his	0.0±0.0	7.8±2.0	6.3±0.0	0.1±0.0	0.1±0.0	0.1±0.1	3.5±0.0	7.0±0.0	7.3±0.6
ile	5.2±0.0	6.0±0.6	5.7±0.3	3.9±0.0	2.3±0.2	2.5±0.1	4.0±0.0	3.9±0.1	4.0±0.2
leu	7.9±0.1	8.2±0.0	7.6±0.2	7.3±0.1	7.1±0.3	7.1±0.1	6.9±0.1	7.0±0.1	7.1±0.0
met	2.2±0.2	1.5±0.2	1.6±0.4	0.5±0.0	0.2±0.0	0.1±0.0	6.4±0.0	3.0±0.1	2.8±0.0
phe	3.7±0.3	2.9±0.2	2.8±0.4	2.2±0.1	1.8±0.0	1.7±0.0	11.5±0.2	8.3±1.3	7.7±0.5
pro	2.8±3.2	4.5±0.1	4.7±0.5	7.6±0.1	7.0±0.0	7.8±0.2	5.7±0.0	5.4±0.2	6.0±0.4
ser	5.08±0.0	2.0±0.3	1.6±0.3	6.2±0.1	8.2±0.5	8.3±0.3	4.2±0.0	4.0±1.0	4.3±0.0
thr	4.8±0.1	5.5±0.2	5.6±0.5	3.6±0.1	1.0±0.3	1.4±0.3	3.7±0.0	4.1±0.6	3.8±0.5
val	6.6±0.1	6.3±0.3	6.8±0.4	7.8±0.1	7.0±0.4	7.3±0.4	6.2±0.1	5.8±0.2	5.9±0.1
lys	7.7±1.5	7.2±0.6	7.3±0.4	3.2±0.4	01.1±0.1	1.0±0.1	4.6±0.2	2.2±0.8	2.2±0.0
tyr	4.1±0.7	2.4±0.2	2.3±0.3	5.1±0.3	12.4±0.5	11.0±0.5	6.2±0.2	9.8±1.9	9.0±0.7
glu	13.2±1.8	12.1±0.6	11.3±0.0	10.7±0.6	12.7±0.3	10.8±0.6	8.9±0.5	10.2±0.8	8.5±1.7
trp	1.6±0.1	1.3±0.0	1.6±0.0	2.9±0.1	4.2±0.2	5.3±0.6	0.4±0.0	1.3±0.0	1.6±0.1
cys	0.9±0.7	-	-	1.0±0.0	-	-	2.4±0.1	-	-

Unf. D.A. unfermented dead adults; D.A.1473 dead adults fermented with *L. rhamnosus* 1473; D.A. 285 dead adults fermented with *L. plantarum* 285; Unf. Pup. unfermented puparia; Pup.1473 puparia fermented with *L. rhamnosus* 1473; Pup. 285 puparia fermented with *L. plantarum* 285; Unf. Pre. unfermented prepupae; Pre.1473 prepupae fermented with *L. rhamnosus* 1473; Pre. 285 prepupae fermentedwith *L. plantarum* 285.

Chapter 4

Effect of processing parameters on food-borne spore-forming microorganisms: new insights from experimental modeling

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Manuscript in preparation

Abstract

Food industry must ensure the stability of the products, and this is often achieved by exposing foods to heat treatments that can ensure the absence of pathogenic or spoilage microorganisms. These treatments are different in terms of temperature and duration, and could lead to a loss in nutritional and sensory value. Moreover, some types of microorganisms manage to survive these treatments thanks to the sporification process. The addition of antimicrobials can become necessary, but nowadays consumers are more inclined to natural products, avoiding synthetic and chemical additives. Antimicrobials from plants could be a valuable option and, in this context, a patent concerning an antimicrobial extract from fermented plant substrate was recently tested against foodborne pathogens revealing high antimicrobial activity. The objective of this study was the creation of a model for the evaluation and subsequent prediction of the combined effect of different process and product variables, including antimicrobial addition, on the inhibition and reduction of spore germination of target microorganisms, *Alicyclobacillus acidoterrestris* and *Clostridium pasteurianum*, responsible for spoilage of tomato-based products.

Introduction

Tomato (*Solanum lycopersicum* L.) is one of the most cultivated vegetables in the world, and the global production accounts for around 170 million tons, where China is the largest producer, followed by the United States and India (FAO 2017). In Italy, about 5.5 million tons are intended for processing in the food industry each year (Garofalo et al., 2017). Throughout the supply chain, from harvesting to the final product, large quantities are discarded. These can be either tomatoes that are not compliant for sale because they do not meet quality or appearance criteria, or by-products generated during the processing of the main product. One of the objectives of the European Union is to become a resource-efficient economy, by the adoption of a circular economy (European Commission, 2019). In this perspective, the reduction or repurpose of waste and by-products is promoted. In the tomato industry, peels and seeds represent about 10-30% of the by-products that are generated during processing (Gharbi et al., 2017). These can be used as animal feed or for the recovery of bioactive components such as pectin, oil, fiber and lycopene (Lu et al., 2019). A patent was recently registered by the University of Parma (n. 102019000006815), based on a lactic acid fermentation process of tomato peels and seeds and subsequent extraction, to obtain an extract with antimicrobial activity for food applications (Ricci et al., 2019). Its use has been tested on meat products and it has been shown to be effective against pathogenic microorganisms belonging to *Salmonella* spp., *Listeria monocytogenes*, *Escherichia coli*, *Staphyloccocus aureus*, *Bacillus cereus*, and *Pseudomonas* spp. (Ricci et al., 2021).

Given the nature of the waste, and with a view to a circular economy, the use of this antimicrobial in tomato-based products should be evaluated to facilitate the recovery of resources lost during production. Moreover, considering the nature of the antimicrobial, this finds space in the growing demand from consumers for natural preservatives (Burt, 2004).

In processed products, the food must be subjected to heat treatments that can ensure the absence of pathogenic or spoilage microorganisms. These treatments, depending on the product, are different in terms of temperature and time. However, some microorganisms manage to survive these treatments thanks to the sporulation process, activated when

conditions are unfavorable and allowing dormancy for long periods. Therefore, spore-forming microorganisms can be found in tomato-based products causing off-flavors as principal defects. Among them, *Alicyclobacillus acidoterrestris*, which produces guaiacol that has a medicinal odor, or *Clostridium pasteurianum*, which can cause swelling of the package (Feng et al., 2010; Komitopoulou et al., 1999).

Different factors can influence microbial behavior: the pH of the medium, the heat treatment time to which they are subjected, or the concentration of antimicrobials, if present in the product. To understand the effect of different factors on growth or inactivation, experimental designs were developed and used in applied microbiology studies to evaluate different treatment and processing and using different target microorganisms (Haberbeck et al., 2012; Parra et al., 2004; Cayré et al., 2003; Silva et al., 1999).

The objective of this study was the building of a model for the evaluation and subsequent prediction of the combined effect of different process and product variables on the microbiological stability of possible tomato-based formulations. In particular, we evaluated the effectiveness of the patent antimicrobial n. 102019000006815 on the inhibition and reduction of spore germination of *Alicyclobacillus acidoterrestris* and *Clostridium pasteurianum,* which often contaminate tomato-based products.

The approach proposed allowed us to identify the combination of optimal parameters for a reduction of these microbial targets in heat treatment. Specifically, for each microbial target, an experimental design that took into account the effect of five factors on the inactivation and germination capacity of the spores was set up. These factors were identified in heat treatment time, water activity (a_w), pH, antimicrobial concentration and spore concentration.

Materials and methods

***Alicyclobacillus acidoterrestris* LMG 16906**

Alicyclobacillus acidoterrestris is one of the target microorganisms, and the strain LMG 16906 was purchased from the Belgian Coordinated Collection of Microorganisms (BCCM / LMG) in freeze-dried form. The culture was revitalized three times with 3% (v/v) inoculum in the *Alicyclobacillus acidoterrestris* Medium (AAM) and incubated at 45 °C for 24 h. AAM is

composed by (i) CaCl2.7H2O, 0.25 g; MgSO4.7H2O, 0.5 g; (NH4)2SO4, 0.2 g; yeast extract, 2g; glucose, 5 g; KH2PO4, 3 g; and distilled water, 1 L ,adjusted to pH 4.0 with H2SO4; (ii) 1 mL of trace elements solution (CaCl2.2H2O, 0.66 g; ZnSO4.7H2O, 0.18 g; CuSO4. 5H2O, 0.16 g; MnSO4.4H2O, 0.15 g; CoCl2.6H2O, 0.18 g; H3BO3, 0.1 g; Na2MoO4.2H2O, 0.03 g; distilled water, 1 L).

Spores were obtained by optimizing some protocols present in the literature (Murakami et al., 1998; Murray et al., 2007; F. V. M. Silva & Gibbs, 2001). Briefly, after revitalization *Alicyclobacillus acidoterrestris* LMG 16906 was grown for seven days in AAM broth at 45 °C. One hundred microliters of the culture broth were inoculated on the surface of the modified AAM agar medium, prepared by adding 50 mg/L of manganese chloride tetrahydrate to AAM, and incubated at a suboptimal temperature of 43 °C for 10 days +/- 1. The presence of spores with respect to the vegetative cells was periodically checked at optical microscope (Olympus System Microscope Models BX51), and when it reached about 90%, the spores were collected. An aliquot of Ringer (Oxoid, UK) was inoculated onto the plates and by gently scraping the spore solution was collected, centrifuged (4000 rpm - 20 min - 4 °C) three times by suspending in Ringer and then stored at -20 °C.

The sporal titer (Log cfu/mL) of the solution was evaluated by plate count following a thermal shock carried out at 80 ° C for 10 minutes to facilitate the activation of the spores and the inactivation of any vegetative forms. The spore solution was decimal diluted sterile water with 0.1% peptone, dilutions were plated in AAM agar medium, and plates were incubated at 45 °C for 48 h.

***Clostridium pasteurianum* LMG 3285**

Clostridium pasteurianum was the second target species considered. Strain LMG 3282 was purchased from Belgian Coordinated Collection of Microorganisms (BCCM / LMG) in freeze-dried form, and it was revitalized in the optimal Reinforced Clostridium Medium (RCM) (Oxoid, Uk) for 7 days +/- days in anaerobiosis (AnaeroGen, Oxoid Ltd) at 37 °C. Spores were obtained following the protocol of Feng et al. (2010): one hundred microliters of the revitalized culture were inoculated on Potato Dextrose Agar (PDA) (Oxoid, UK),

modified with the addition of 0.75% (w/v) of calcium carbonate the modified agar medium, and incubated at a suboptimal temperature of 30 °C in anaerobiosis for thirty days.

The presence of spores with respect to the vegetative cells was periodically checked at the optical microscope. When they were mainly present, 5 mL of sterile water were added to the agar plates, and the spores were collected after gently scraping. Spore solution was centrifuged and washed (5000 g - 15 min - 4 °C) with sterile water three times, resuspended in water/ethanol (50/ 50 v/v) and left at room temperature (approx. 22 °C) for one hour. Washing by centrifuge was repeated five times with sterile water, and then the spore solution was stored at 4 °C.

The sporal titer (Log cfu/mL) of the solution was evaluated by plate count. Before the count, the solution was thermal shocked at 80 °C for 10 minutes to induce the activation of the spores and the killing of any vegetative forms. The solution was decimally diluted in Ringer solution, and dilutions plated in RCM agar medium. The plates were then incubated at 37 °C in anaerobic conditions for 48 h.

Design of Experiments (DOE)

The experiments were designed according to a mathematical model using MODDE Pro v12.0.1 software (MKS Umetrics, Umeå, Sweden). The model was planned around five quantitative factors: pasteurization time, a_w, pH, antimicrobial concentration and spore concentration. The responses measured are the percentage of germination and the percentage of inactivation of the spores. Germination is defined as the percentage of spores that manage to germinate, after pasteurization. Inactivation was considered as the percentage of spores killed or damaged after heat treatment, therefore no longer able to germinate. The response surface model was elaborated with a Central Composite Full factorial design (CCF). This type of design requires to set three levels of analysis for each parameter: an average value, a maximum and a minimum value for a total of 28 experiments to be carried out in duplicate for each target strain. The values set for each parameter are summarized in Figure 1.

Figure 1. Values of the quantitative factors

Name	Abbreviation	Units	Type	Use	Settings
Temperature	Temp	°C	Quantitative	Constant	90
Time	Min	min	Quantitative	Controlled	4 to 10
Water activity	Aw		Quantitative	Controlled	0,94 to 0,98
pH	pH		Quantitative	Controlled	4 to 5
Antimicrobial	Ant	%	Quantitative	Controlled	0 to 2,4
Spores	Spo	CFU/ml	Quantitative	Controlled	1e+04 to 3,48e+05

The a_w ranges were chosen to reflect the values of the tomato-based products on the market (tomato paste aw 0.94 – tomato sauce 0.98). Also for pH, we choose a range that could reflect the tomato-based product. The three different levels of pasteurization time were chosen starting from 10 minutes that is the time usually set for pasteurization. The study of each model was performed in the optimal culture medium of the target microorganism. The pH of the solution was modified with Hydrochloric acid (HCl), while the a_w was modified by adding sodium chloride (Sigma-Aldrich, St Louis, MO, USA) and glycerol (Sigma-Aldrich, St Louis, MO, USA) at a known concentration.

The set of experiments was prepared by adding to the medium the antimicrobial, and then the spores of the target microorganisms after activation with thermal shock (80 °C - 10 min), both at the different concentrations according to the model. Once inoculated, the heat treatment at 90 °C was applied considered three different times according to the model (4 - 7-10 min). The samples were then incubated at the optimal temperature for one week, after which the percentage of germination and the percentage of inactivation of the spores were evaluated by plate count on selective medium.

Figure 2. DoE of *A. acidoterrestris*

	Exp No	Exp Name	Run Order	Incl/Excl	Temperature	Time	Water activity	pH	Antimicrobial	Spores
1	1	N1	3	Incl	90	4	0,94	4	0	348000
2	2	N2	50	Incl	90	10	0,94	4	0	10000
3	3	N3	46	Incl	90	4	0,98	4	0	10000
4	4	N4	24	Incl	90	10	0,98	4	0	348000
5	5	N5	41	Incl	90	4	0,94	5	0	10000
6	6	N6	11	Incl	90	10	0,94	5	0	348000
7	7	N7	4	Incl	90	4	0,98	5	0	348000
8	8	N8	1	Incl	90	10	0,98	5	0	10000
9	9	N9	2	Incl	90	4	0,94	4	2,4	10000
10	10	N10	16	Incl	90	10	0,94	4	2,4	348000
11	11	N11	19	Incl	90	4	0,98	4	2,4	348000
12	12	N12	12	Incl	90	10	0,98	4	2,4	10000
13	13	N13	33	Incl	90	4	0,94	5	2,4	348000
14	14	N14	30	Incl	90	10	0,94	5	2,4	10000
15	15	N15	14	Incl	90	4	0,98	5	2,4	10000
16	16	N16	42	Incl	90	10	0,98	5	2,4	348000
17	17	N17	28	Incl	90	4	0,96	4,5	1,2	100000
18	18	N18	52	Incl	90	10	0,96	4,5	1,2	100000
19	19	N19	5	Incl	90	7	0,94	4,5	1,2	100000
20	20	N20	37	Incl	90	7	0,98	4,5	1,2	100000
21	21	N21	31	Incl	90	7	0,96	4	1,2	100000
22	22	N22	32	Incl	90	7	0,96	5	1,2	100000
23	23	N23	34	Incl	90	7	0,96	4,5	0	100000
24	24	N24	9	Incl	90	7	0,96	4,5	2,4	100000
25	25	N25	49	Incl	90	7	0,96	4,5	1,2	10000
26	26	N26	44	Incl	90	7	0,96	4,5	1,2	348000
27	27	N27	56	Incl	90	7	0,96	4,5	1,2	100000
28	28	N28	53	Incl	90	7	0,96	4,5	1,2	100000
29	29	N29	35	Incl	90	7	0,96	4,5	1,2	100000

Figure 3. DoE of *C. pasteurianum*

	Exp No	Exp Name	Run Order	Incl/Excl	Temperature	Time	Water activity	pH	Antimicrobial	Spores
1	1	N1	3	Incl	90	4	0,94	4	0	1e+06
2	2	N2	50	Incl	90	10	0,94	4	0	10000
3	3	N3	46	Incl	90	4	0,98	4	0	10000
4	4	N4	24	Incl	90	10	0,98	4	0	1e+06
5	5	N5	41	Incl	90	4	0,94	5	0	10000
6	6	N6	11	Incl	90	10	0,94	5	0	1e+06
7	7	N7	4	Incl	90	4	0,98	5	0	1e+06
8	8	N8	1	Incl	90	10	0,98	5	0	10000
9	9	N9	2	Incl	90	4	0,94	4	2,4	10000
10	10	N10	16	Incl	90	10	0,94	4	2,4	1e+06
11	11	N11	19	Incl	90	4	0,98	4	2,4	1e+06
12	12	N12	12	Incl	90	10	0,98	4	2,4	10000
13	13	N13	33	Incl	90	4	0,94	5	2,4	1e+06
14	14	N14	30	Incl	90	10	0,94	5	2,4	10000
15	15	N15	14	Incl	90	4	0,98	5	2,4	10000
16	16	N16	42	Incl	90	10	0,98	5	2,4	1e+06
17	17	N17	28	Incl	90	4	0,96	4,5	1,2	100000
18	18	N18	52	Incl	90	10	0,96	4,5	1,2	100000
19	19	N19	5	Incl	90	7	0,94	4,5	1,2	100000
20	20	N20	37	Incl	90	7	0,98	4,5	1,2	100000
21	21	N21	31	Incl	90	7	0,96	4	1,2	100000
22	22	N22	32	Incl	90	7	0,96	5	1,2	100000
23	23	N23	34	Incl	90	7	0,96	4,5	0	100000
24	24	N24	9	Incl	90	7	0,96	4,5	2,4	100000
25	25	N25	49	Incl	90	7	0,96	4,5	1,2	10000
26	26	N26	44	Incl	90	7	0,96	4,5	1,2	1e+06
27	27	N27	56	Incl	90	7	0,96	4,5	1,2	100000
28	28	N28	53	Incl	90	7	0,96	4,5	1,2	100000
29	29	N29	35	Incl	90	7	0,96	4,5	1,2	100000

Evaluation of spore germination and inactivation

After a week of incubation, the concentration of vegetative cells, the quantity of spores that managed to germinate, was evaluated by plate count. Samples were decimally diluted in Ringer and dilutions were plated on the optimal agar medium (AAM and RCM, for *A. acidoterrestris* and *C. pasteurianum* respectively). The percentage of germination was calculated based on the initial inoculation of spores and final concentration of cells, following the formula: $(n_t/n_0)*100$, where n_t is the vegetative cells germinate after one week of incubation and n_0 is the initial inoculum of spores.

The samples were then analyzed to assess the inactivated spores. Samples were thermal shocked at 80 °C for 10 min to kill vegetative cells and activate the remaining spores, subsequently plate count on optimal medium was carried out. The percentage of inactivated spores was calculated starting from the initial concentration, following the formula: $100 - (n_t/n_0)*100$, where n_t is the spore load after one week of incubation and n_0 is the initial inoculum of spores.

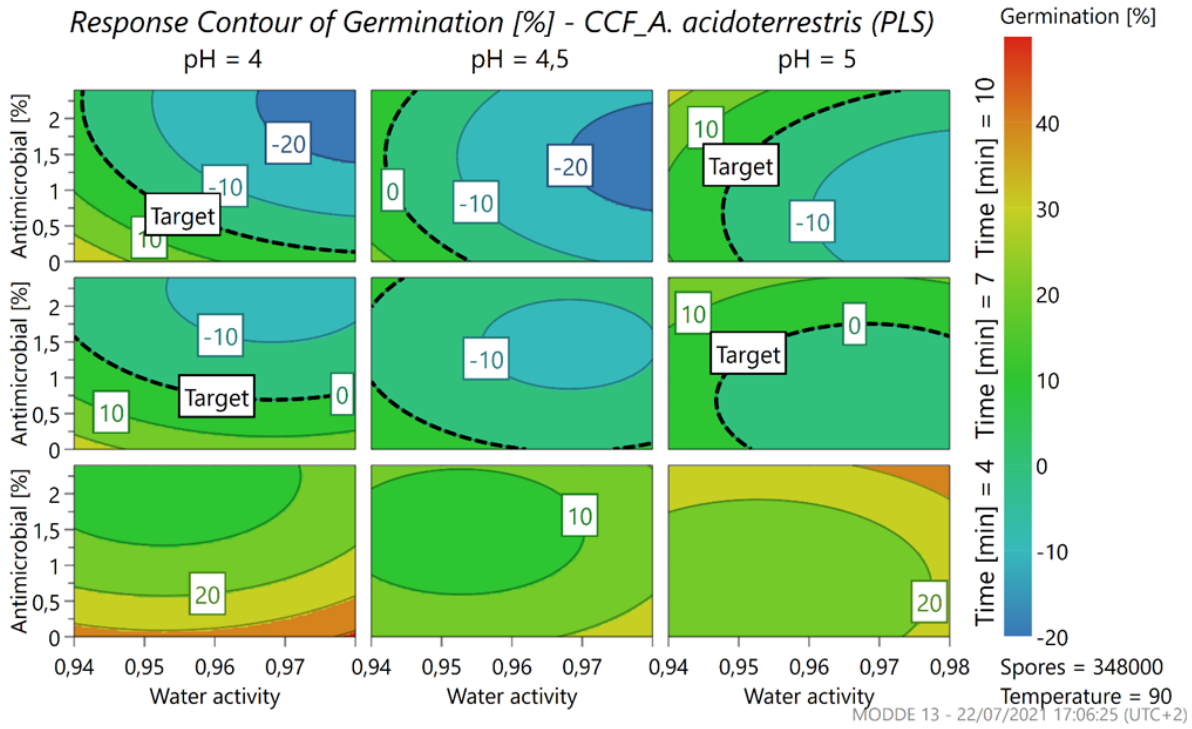
Results

***Alicyclobacillus acidoterrestris* LMG 16906**

From the analysis of the 58 experimental conditions, the software created a response surface that shows the germination and inactivation trend of the spores, according to the variations of the different parameters. To better understand the variations of response, we keep the concentration of the inoculum fixed at the maximum level (5,5 Log cfu/ml). Six different contour plots, representing the response surface, were thus obtained (Fig. 4 and 5), which change depending on the duration of the heat treatment and the pH of the medium. To better visualize the area of interest, the target to reach was established at 0% for germination and 100% for inactivation (optimal response).

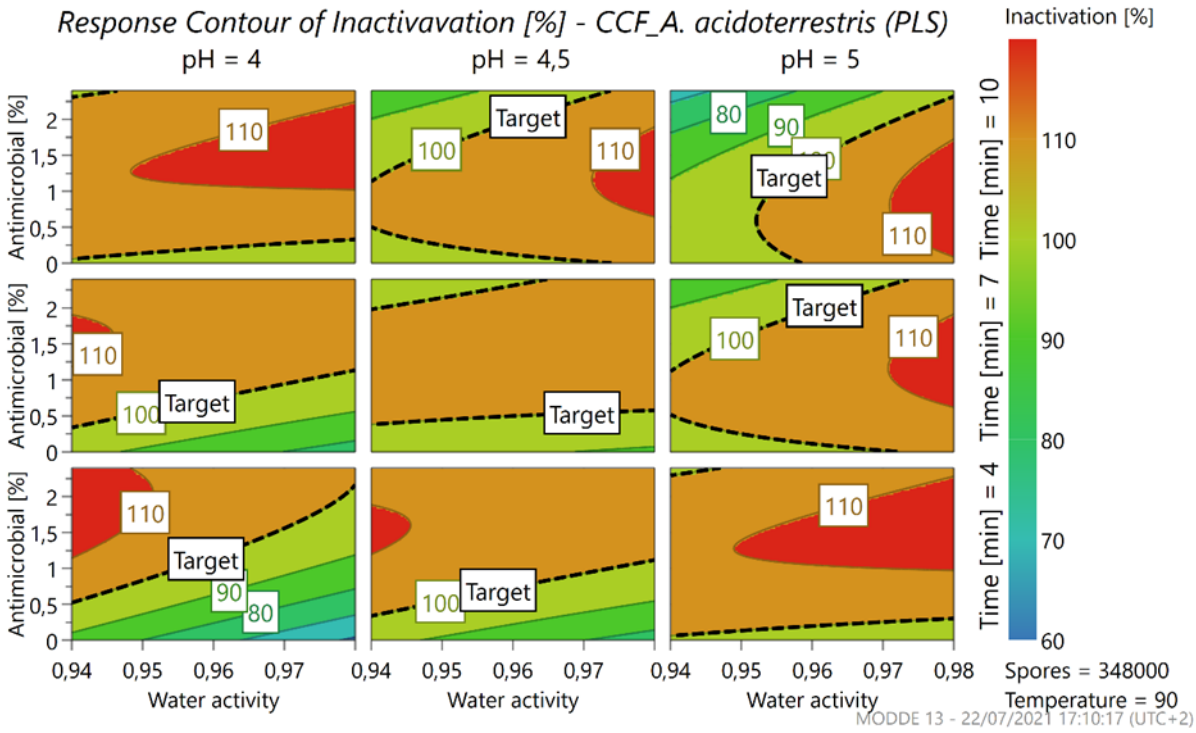
Looking at the graph corresponding to the percentage of germination (Figure 4), the response changes mainly as a function of the heat treatment time, pH values of the medium and quantity of antimicrobial added to products.

Figure 4. Contour plot representing the percent of germination of *A. acidoterrestris*, at different levels of parameters.



Starting from the top of the graph, the first row shows samples treated for ten minutes, and highlight how the circle delimited by the target covers all the different range with different water activity. This means that in general, after one week of incubation, 0% germination is recorded when 10 minutes of heat treatment at 90°C is set up. However, when pH of the solution becomes less acidic the circle start to restrict, but the percentage of germination still remains within 10%. Interestingly, the addition of the antimicrobial leads to similar results (0% germination) even at shorter pasteurization times (7 min). The last line of the graphs corresponds to the shorter heat treatment time (4 min) and highlights that the total absence of germination is not possible but can be minimized by adding about 1% of antimicrobial. Also regarding spore inactivation (Figure 5) pH and treatment time are the parameters that affect the response, together with the a_w of the medium.

Figure 5. Contour plot representing the percent of spore inactivation of *A. acidoterrestris*, at different levels of parameters.



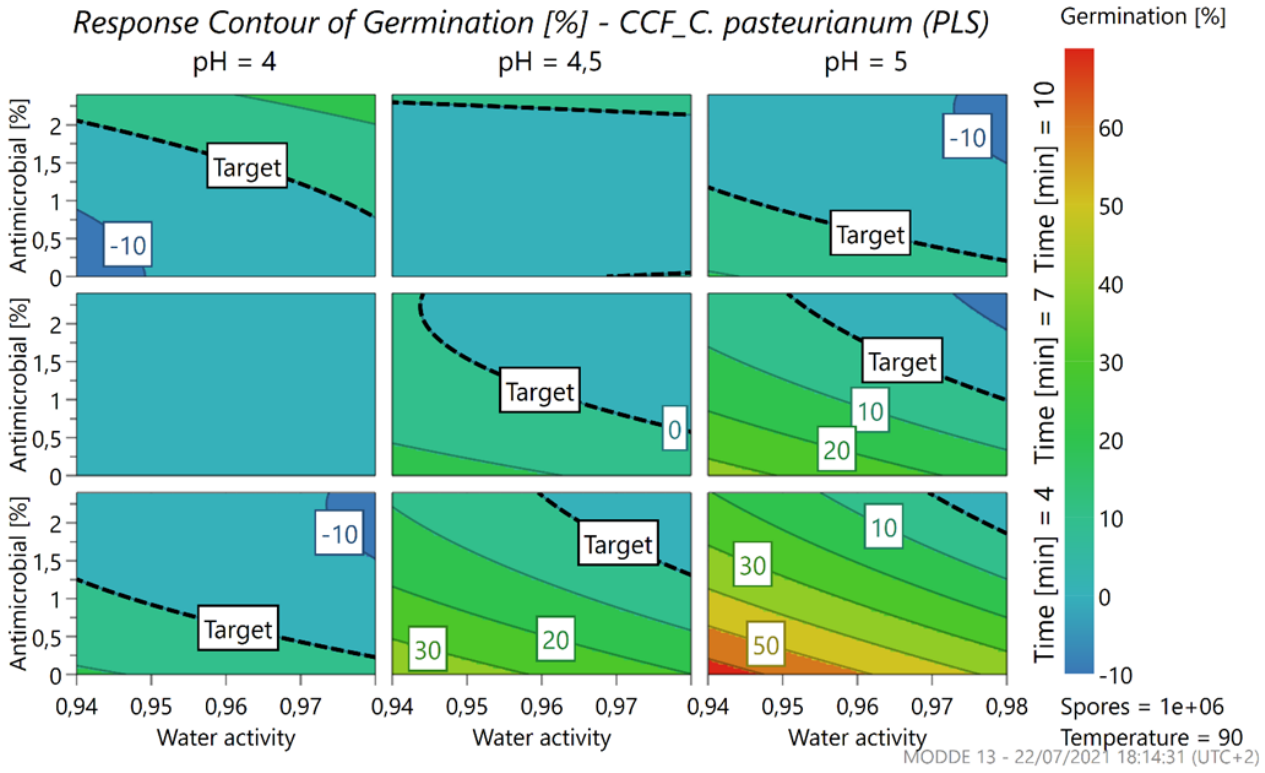
Observing the first column that corresponds to the samples inoculated in the media at pH 4, 100% inactivation is generally guaranteed for all a_w values but, as the a_w increases, the circle start to narrow upwards. This highlights how the inactivation objective is guaranteed thanks to the addition of the antimicrobial. This condition also allows the treatment time to be reduced up to 4 minutes.

***Clostridium pasteurianum* LMG 3285**

Also for this species, the analyzed samples were 58 and, keeping the concentration of the inoculum unchanged at the considered maximum level of 6 Log cfu/ml, the software created a response surface.

C. pasteurianum exhibited similar behavior to *A. acidoterrestris*. The plots presented in Figure 6 show, also in this case, that the main factors influencing the percentage of germination are pH, heat treatment time and antimicrobial addition.

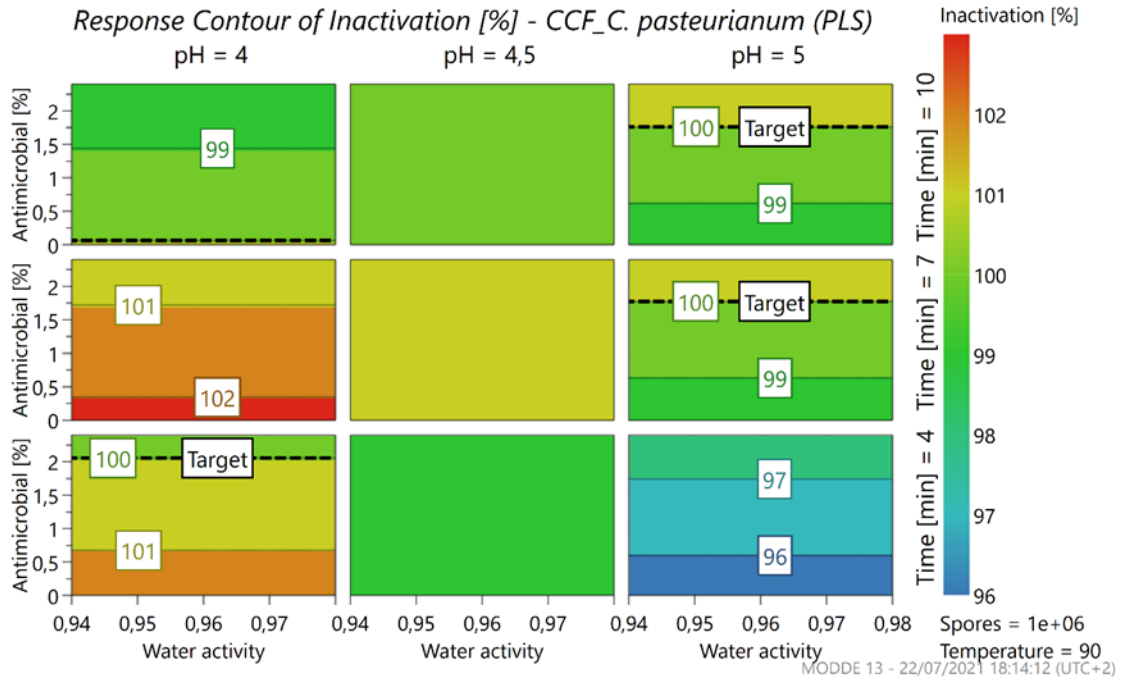
Figure 6. Contour plot representing the percent of germination of *C. pasteurianum*, at different levels of parameters.



In general, at the lowest pH considered (pH 4), the percentage of germination remains low even with reduced treatment times (4 minutes). At pH 5 and with treatment times of less than 10 minutes, the target circle narrows but it is still possible to obtain a low percentage of germination with the addition of the antimicrobial.

Figure 7 highlights that the percentage of inactivated *C. pasteurianum* spores is very high and close to 100% in almost all cases. In fact, observing the box at the bottom right (best condition for growth with higher pH and low treatment times), even in the absence of antimicrobials, an inactivation of around 96% is obtained.

Figure 7. Contour plot representing the percent of spore inactivation of *C. pasteurianum*, at different levels of parameters.



As for the germination, the highest pH considered (pH 5) leads to reduced effectiveness of heat treatment. However, the target of 100% inactivation is reached with the addition of antimicrobial extract.

Discussion

A current challenge for the food industry is to ensure the stability of the product, trying to increase its shelf life while minimizing the negative effects of heat treatments on organoleptic properties. Therefore, the addition of antimicrobials becomes often necessary even if consumers are more attracted towards products labeled as "natural", frowning on chemicals and preservatives in general (Tajkarimi et al., 2010). In this debate, antimicrobials obtained from fermented plant substrates, like tomato by-products, could represent a valuable strategy for food industry. Moreover, in a circular economy perspective, the application of this antimicrobial in a tomato-based product could close the loop of primary resource utilization and reduction of by-products. In this study, the effectiveness of such a natural antimicrobial to reduce the percentage of germination and the ability to inactivate spores of spoilage target microorganisms was evaluated. Results from the two DoE showed activity of the antimicrobial against spores of both species considered (*A. acidoterrestris* and *C. pasteurianum*) highlighting the pH, time of treatment and a_w as the parameters that most influence the response as reported by Silva et al. (1999).

Bacterial endospores are characterized by their need for special requirements to initiate germination and outgrowth. Among these needs are optimum a_w values and nutrient availability (Beuchat, 2017). As reported by Silva and Gibbs (2001), the condition of the media strongly affects heat resistance of the spores, and one of the most significant variables is the pH that has a negative correlation respect to the germination process, and it depends on the temperature used for thermal inactivation (Silva et al., 1999). According to numerous studies, bacterial spores become more heat resistant when water activity is reduced. In the present research, this trend referred to germination was recorded for both the target species. Indeed, we observed that high a_w is associated with an increase in percentage of germination. As described above, adding antimicrobials could lead to a shorter heat treatment to prevent the germination of *A. acidoterrestris* and *C. pasteurianum*. This finding is important as consumer demand for fresh, under-processed or unpasteurized foods has increased in recent decades due to their high levels of vitamin C, polyphenols and antioxidants deteriorate with treatments at high temperatures (Aneja et al., 2014). Various research in the literature have focused on natural antibacterial activity of essential oils, plant extract, and algae (Bancalari et al., 2020; Martelli et al., 2020; Liu et al., 2017; Pina-Pérez et al., 2017; Takahashi et al., 2004; Yim et al., 2006). Moreover, antibacterial activity of fermented extracts/substrate starting from by-products is poorly reported (Hadj Saadoun et al., 2020; Di Onofrio et al., 2019; Eom et al., 2017).

Extract from fermented tomato by-products used in this study showed an antimicrobial activity, recently reported against food-borne pathogens both for *in vitro* and *in situ* studies (Ricci et al., 2021; Ricci et al., 2020). In this study, the effectiveness towards different microbial targets, and in particular their spores, was also demonstrated. In particular, the antimicrobial tested reduced the ability of germination of spores and/or led to the inactivation of spores. The compounds responsible for this activity have not yet been identified but there are various hypotheses. The activity could derive from the compounds naturally present in substrates, such as polyphenols (Hayouni et al., 2007). Besides, during fermentation, various organic acids or bacteriocins could be produced (Leroy & De Vuyst, 2004). For example, the activity against spore-forming of nisin, a polypeptide produced by

some strains of lactic acid bacteria have been reported (Komitopoulou et al., 1999; Yamazaki et al., 2000).

Conclusion

The control of spoilage microorganisms, in particular spore forming bacteria, represents a key element in the quality control of the processing industries. In tomato industry, two of the major targets to be monitored during the production process are *Alcyclobacillus acidoterrestris* and *Clostridium pasteurianum* because they can deeply alter the final product.

In this study, thanks to the Design of Experiment (DoE) approach, it was possible to verify the influence of various factors on the germination and inactivation of the bacterial spores, and to evaluate the process and product conditions that can guarantee the microbiological stability of the product, possibly reducing the heat treatment time, and as a consequence the cost of the process. In addition, it was possible to study the effect of a new extract recently patented on the spores of microbial targets with the final aim to evaluate its use in tomato commercial products.

The data obtained allow the development of predictive models that can be implemented and managed for the design of new products or treatments. In our opinion, the creation of this model represents a useful tool for tomato processing companies but also for other companies that have to keep the same targets under control (eg juice industries). Indeed, with the predictive function of the software it is possible to evaluate the different responses (germination, inactivation) according to the type of product to be considered (e.g. according to specific a_w, pH). Future studies could be directed towards the validation of these models in commercial products, or the implementation of these data with product shelf life studies or with sensory data to verify the impact of the addition of the antimicrobial on the characteristics of the product.

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Conclusion

The present research enabled to improve the knowledge about the possibilities offered by LAB for agri-food by-products enhancement, focusing on the production of natural flavors and antimicrobial compounds. The novelty of this project was represented by the application of lactic fermentation on new by-products not yet explored (i.e exhausted vanilla beans, insects, melon pulp and orange pomace), the utilization of a new approach, the DoE, which permits to study multiple factors together and acquire differents data about the process and the starters (i.e work on exhausted vanilla beans and work on antimicrobial against spore forming bacteria). Moreover, beyond the knowledge acquired on the possibility to ferment different substrates, this study investigates and brings out the use of co-cultures as a starter for lactic fermentation (i.e works on okara).

The use of microbial cultures offers several advantages over traditional methodologies, such as the possibility to label the products as "natural," thus making them more attractive for consumption, with market acceptability. Studies conducted on fermentation of different matrices have allowed us to evaluate how the sensory profile of the product changes and which molecules are obtained. In the case study with melon and orange by-products, we observed that thanks to the unique enzymatic portfolio of *L. rhamnosus* 1473, the substrates were modified leading to the production of volatile compounds with interesting and positive aroma, like floral, herbal green, and fruity notes. In the fermented samples, we recorded an increase of β-linalool, trans geraniol in orange by-products, or 2-hexenal, butanoic acid, and β-damascenone in melon by-products using simple distillation.

Our study on okara by-product demonstrated that lactic fermentation is a valid strategy for improving the features of this substrate. In this case, we evaluated three different strains observing the adaptation of the majority to the substrates, and the LAB co-cultures that are not widely studied. The co-culture containing *L. acidophilus*, *L. rhamnosus*, and *P. acidilactici* was the best starter candidate due to its ability to significantly modify the aromatic and polyphenolic profile of raw material. Besides the optimal growth performance, a decrease of off-flavor (hexanal, nonanal) and a large conversion of isoflavones in their aglycone forms were obtained. Moreover, a notable production of LAB-derived metabolites such as indol-

3-lactic, phenyllactic and p-hydroxyphenyllactic acids, that can exert a human biological activity or antimicrobial activity, was observed.

To evaluate the best process parameters for different microbial strains to be used as starters, we adopted the use of mathematical models for the fermentation of exhausted vanilla beans. This strategy allows us to obtain general information on fermentation. Indeed, results indicate that a higher bacterial load is expected at lower than optimal temperatures and in a short time. Unlike what was expected, the addition of glucose is required only for a few strains. The samples fermented with co-cultures (*L. rhamnosus* and *L. plantarum*) were highlighted also in this case as the best one, with persistent sweet, sugared almonds, panna cotta, and pudding notes. Moreover, from the instrumental SPME-GC-MS analysis, it was observed that in the samples fermented with the co-culture the content of vanillin and guaiacol is higher than in all the others suggesting that fermentations release these compounds as a consequence of the metabolic activity of the strains.

Considering the scientific and commercial interest in discovering novel antimicrobials, part of PhD project was directed towards the production of new antimicrobial compounds from fermented substrates and to the study of implementation in a commercial product.

As the review in chapter three illustrated, insects are a valuable frontier in food and feed applications as they have underestimated amount of antimicrobial compounds present in their body components. Large quantities of waste are generated in the mass rearing of insects, and we want to evaluate if it is possible their possible reuse using fermentation. In particular, we studied the waste deriving from *Hermetia illucens* breeding, represented by puparia and dead adult flies. This work aimed to answer questions whether LAB can grow and ferment fractions obtained during the rearing of *H. illucens* and whether it is possible to produce high added value molecules during fermentation. As a whole, puparia and adults are fermentable and after fermentation they show antimicrobial activity. The preliminary analysis on LAB fermented insect biomasses showed a shift in lipid and protein composition induced by LAB, suggesting that the fermentation caused important changes in the molecular composition of the biomass analyzed.

As a final part of the project we studied, with predictive and optimization models the use of an antimicrobial obtained from the fermentation of plant by-product. The study was conducted in vitro with spore-forming bacteria that contaminate tomato products. Using an experimental design, where the ranges of five factors (pasteurization time, aw, pH, spore concentration, and antimicrobial) were modified to represent a wide range of tomato commercial products, it was possible to evaluate the effectiveness of the antimicrobial and the interaction between factors that could allow the use of shorter heat treatment. Moreover, this type of approach has allowed the development of a predictive model that can be exploited by the company for the study of new products.

About the author

Jasmine Hadj Saadoun was born on 20th April 1990 in Parma, Italy. She got bachelor's degree in Gastronomic Sciences at the University of Parma discussing the thesis "Evaluation of 5 cultivars of *Pistacia vera* through sensory analysis and consumer tests". To obtain the data she worked at the Centro Nazionale delle Ricerche (CNR) – Istituto di Biometerorologia (IBIMET) in Bologna. She persecuted her study with the Master's degree in Food Control and Safety at the University of Modena and Reggio Emilia, discussing the thesis "Evaluation of different biological parameters of *Hermetia illucens* reared on different by-products". The project continues for another year, during the research fellow at the Applied Entomology laboratory in Reggio Emilia. In November 2018, Jasmine passed the section for the Doctoral School in Food Science at the University of Parma, under the supervision of Prof. Camilla Lazzi and Prof. Gianni Galaverna. During the three years of her PhD she focused on fermentation by lactic acid bacteria on different matrices with different objectives, from obtaining antimicrobial molecules to studying the modification of the volatile and nutritional profile, to add value to waste and thinking about a possible reuse. The results achieved in the three years of the PhD are described in this thesis.

Scientific activity

Original papers

Ricci, A., Marrella, M., Hadj Saadoun, J., Bernini, V., Godani, F., Dameno, F., Neviani, E., & Lazzi, C. (2020). Development of lactic acid-fermented tomato products. Microorganisms, 8(8), 1192.

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Hadj Saadoun, J., Sogari, G., Bernini, V., Camorali, C., Rossi, F., Neviani, E., Lazzi, C. A critical review of intrinsic and extrinsic antimicrobial properties of insects. Submitted to Trends in Food Science and Technology.

Poster Presentation

Hadj Saadoun, J., Luparelli, A. V., Caligiani, A., Macavei, L. I., Maistrello, L., Neviani, E., Galaverna, G., Sforza, S., & Lazzi, C. Antimicrobial Biomasses from Lactic Acid Fermentation of Black Soldier Fly Prepupae and Related By-Products. World Microbe Forum, An ASM & FEMS collaboration, Online Worldwide, 20-24 June 2021.

Hadj Saadoun, J., Luparelli, A. V., Caligiani, A., Macavei, L. I., Maistrello, L., Neviani, E., Galaverna, G., Sforza, S., & Lazzi, C. Antimicrobial activity of fermented insect: a study of insect-derived waste biovalorization through solid state fermentation. 5th International Conference on Microbial Diversity, Catania, 25-27 September 2019.

Hadj Saadoun J. Fermentation as a strategy to produce high value-added compounds from by-products and waste. 24th Workshop on the Developments in the Italian PhD Research on Food Science, Technology and Biotechnology. University of Firenze, Firenze 11-13 September 2019.

Oral presentation

Hadj Saadoun J. Fermentation as a strategy to produce high value-added compounds from by-products and waste. First Virtual Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology. University of Palermo, September 14th-15th, 2021.

Grant and Awards

Winner of a FEMS Grants to participate in the World Microbe Forum (an ASM & FEMS collaboration) conference, 20-24 / 06/2021, Online Worldwide.

Winner of the ICFMH - Best Poster award for the best poster at the 5th International Conference on Microbial Diversity, 25-27 / 09/2019, Catania (IT)