UNIVERSITY OF NAPOLI – FEDERICO II

DEPARTMENT OF PHARMACY

Ph.D. THESIS IN
PHARMACEUTICAL SCIENCE

New Nutraceuticals from natural matrices by products to prevent diabetes

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Research end points

Starting from the impact that the metabolic syndrome, which includes a cluster of health disorders like obesity, diabetes and cardiovascular diseases,and based on the consideration that these conditions are very closelyrelatedto dietary foods habits, there is the need to develop a sustainable and novel nutraceutical approach as a complementary and/or alternative to the conventional pharmacologic treatment. The main goal is the return to euglycemic conditionandrepresent a relevant challenge exploited in this thesis.

Both quality and safety of edible natural products are closely related to the compliance with the principles of good agricultural practice at every step of the production. Microfungi are one of the main threat in crop cultivation. The secondary metabolites produced can affect also the phytocomplex and/or the food characteristics, since technological treatments do not affect them.

Based on this, my research objectives can be divided in four main steps:

- Use probiotics as biocontrol agents for a safe approachto nutraceutical preparation. A case study based on tomatoes is presented.
- Project and study new nutraceuticals based on different vegetal matrices by products targeting the diabetes health condition.
- Highlight *in vitro* and *in vivo* the efficacy of these new nutraceutical formulationsonglyco-metabolic parameters.
- Formulate a tomatoes puree lacto-fermented with *Lactobacillus* spp.to improve the amount of bioactive compounds with hypoglycemic beneficial health effect.

Abstract

The control of glucose homeostasis is the main goal for both the prevention and management of type 2 diabetes (TD2) and pre-diabetes. While T2D is an irreversible condition, pre-diabetes is not.The objective of this research thesis has been to approachthe pre-diabetes conditionand develop from natural sourcesa successful preventive and therapeutic strategy by increasing in the dietthe levels of active compounds capable to control the glycemia. Currently, a great interest has been focused on the valorization of agro-food byproducts, since these matrices could be a source of essential bioactive substance useful to the nutraceuticaland food supplements industry. Supporting the perspective of food as medicine, the aim of the thesis has beento provide an innovative alternativetoolthrough the determination of various phytochemical compounds (e.g. carotenoids, polyphenols, terpenoids,abscisic acid)capable to prevent and/or to control the diabetes.

While drugs are strictly regulated, the controlsonfood supplements and nutraceuticals arelimited, in these regardthe vegetal origin of phytocomplexes and possible mycotoxins occurrence may affect the processes of extraction and concentration of the active principles, representing a critical point to be monitored to provide safe and mycotoxins free nutraceuticals and foodsupplements.The effect of different *Lactobacillus*strainshas been evaluatedas a suitable alternative of biocontrol to protect crops, in particular tomatoes, and a case study is presented. The possibility to use *Lactobacillus* to avoid/prevent the presence of secondary metabolites has been assessed. The following steps of this Thesis regarded the characterization, analysis, purification, formulation, and*in vitro* and *in vivo* tests of phytocomplexes from differet vegetal matrices. For this purpose, two double blind, randomized, clinical trials have been realized to verify the efficacy of novel nutraceutical formulations from: i)nectarines (NecP); ii)tomato peels (TP); iii) olive leaves (EOL), on glycaemic and insulinemic responses, in postprandial glycaemia (PPG), impaired fasting glucose (IFG), and impaired glucose tolerance (IGT) conditions. All the three formulations significantly lowered the 30 min glucose plasma peak (p <0.05). NecP and TP also significantly lowered the 30 min insulin plasma peak (p<0.05).

In particular, the phytohormone abscisic acid, identified for the first time in our laboratory in nectarines thinning waste (NecP),has been found to be effective in ameliorating glyco-metabolic compensation and in reducing inflammatory status (Hs-CPR) in patients with IFG or IGT.

As a part of this Thesis, lacto-fermented tomatoes pomace puree enriched with two strains ofprobioticshas been formulatedto evaluate, after the fermentation, the *in vitro*and*in vivo*potential through the Oral Glucose Tolerance Test (OGTT) to verify the beneficial effect in glucose metabolism.The data obtained by HPLC-DAD/Spectrometric-UV showed how the fermentation may impact positively on bioactive compound content, enzymatic response, and decrease the glucose concentration.

All these results are promising for further investigation in the clinical research area.

Introduction

1.1 Nutraceutical an overview:definition, benefits, recent findings

Way before the quote by Hyppocrates: ‘let the food be thy medicine and medicine be the food’, Ayurveda medicine recognized the beneficial influence of food and plants consumption intake on human health (Tiwari and Morya 2018). The last decadesthe role of dietary active components become an important focus of research and have increased the awareness of consumers about diet and proper nutrition.In this context, a new food derived category that has emerged:the"nutraceuticals". This brings the mind back to the Stephen DeFelice definition of nutraceutical: ‘food or part of a food that provides medical or health benefits, including the prevention and/or treatment of a disease’ (DeFelice 1995). In the recent years the original definition has been reformulated into: “nutraceuticals are defined as the phytocomplex if they derive from a food of vegetal origin, and as the pool of the secondary metabolites if they derive from a food of animal origin, concentrated and administered in the more suitable pharmaceutical form” (Santini et al. 2018).

Recently, dietary recommendations have suggested an increase in the consumption of foods rich in phytochemicals(Liu 2003) like the ones which contain a significant amounts of phenolic compounds. These last are contained mainly in fruits, vegetables, wines, and teas, which provide benefits to human health and play an important role in preventing chronic diseases, particularly the diabetes health condition. The study of the phytocomplexes from these vegetal matrices targetsthescope of my research.

Since the diabetes is a growing health issue worldwide, nutraceuticals can be a challenge for the future of prevention and therapy of this disease, as well as a triggering tool in the medicine area to face pathological, chronic, long-term, asymptomatic diseases in subjects who do not qualify for a pharmacological therapy.

This is proven true by the number of nutraceuticals formulation patents, which has grown exponentially, indicatingthe key role attributed to nutraceuticals as a possible therapeutic approach before the pharmaceutical treatment (Yeung et al. 2018, Daliu et al. 2019).

As shown in Table 1, a large number of nutraceuticals in the last 10 years are addressed to health conditions related to the metabolic syndrome, especially the diabetes andits possiblecomplications,to favorably modulate a number of biochemical endpoints.

Target pathology	Lead substances	Novel Patents
Diabetes	Lycopene	US7887848B2 (2011)
	lipoic acid	WO2010110640A1 (2010)
	dietary fibers	US7943163B2 (2011)
	essential oil	US2012/0213756 A1 (2012)
	anthocyanins	US8536224B2 (2013)
	carotenoids	EP 1 997 500 B1 (2013)
	alkaloid(berberine)	US9192180B2 (2015)
		US9937220B2 (2018)
Cholesterol (HDL/LDL) and Cardiovascular diseases	Polyphenols	US20140314729A1 (2014)
	proanthocyanidin B2 catechins	US20140314729A1 (2014)
	omega-3vegetal sterols	WO2014/014766 (2014)
	Q10coenzime	US9579356B2 (2017)
	organosulfuric compounds	
Weight management	linoleic acid	WO2012141454 (2012)
	capsaicin	WO2012094739 (2012)
	glucomannan	US 20120027787 (2012)
	chitosan	US8524980B2 (2013)
	L-carnitine	US8481072B2 (2013)
	triterpenoids (cucurbitane)	US 8945608 (2015)
		WO2016073326A2 (2016)
Joint Tissue and arthritis	glucosamine sulfatechondroitinesulfate hydrolyzedcollagen	US20140309203A (2014)
Immunomodulator	ginsenosides	WO2010074553A1(2010)
	alkamides	US9675538 (2015)
	lignans (arctigenin)	US20160095879A1 (2016)
	polyphenol (curcumin)	
	polysaccharides (ginsan)	
Probiotics Prebiotics	Lactobacillus	WO2011092261A1 (2011)
	Bifidobacterium	US9155766B2 (2015)
	Galacto-oligosaccharide	WO/2015/090349 (2015)

Table 1. Recent patents requested for novel nutraceuticals

*Data were retrieved from the United States Patent and Trademark Office (USPTO, https://www.uspto.gov/), European patent Office (Espacenet, https:// worldwide.espacenet.com), Google Patents (https://patents.google.com/), Global Dossier (https://globaldossier.uspto.gov/#/), Justia Patents (https://patents.justia. com/).

In addition, nowadays the continuous growing interest of consumers for natural source and the problems associated with the use of chemical pesticides and food preservatives, makes it necessary to search for suitable alternatives.The health benefits of fruit and vegetables have been extensively recognized in recent studies. However, some vegetal contaminants, like the mycotoxins, can cause serious problems for humans.Plants and medicinal herbs that are available on the market do not always meet quality and safety standards.While drugs are tightly regulated, the control over nutraceuticals and food supplements is relatively low. Conform with the growing demand and safety request for food derived supplements and functional food,the possible application of *Lactobacillus* and *Bacillus* strains, has been explored as a first step, before the formulation of a nutraceutical.Figure 2 shows a scheme that have been followed in the research of nutraceutical for proactive action in prevention and therapy of selected pathological conditions.

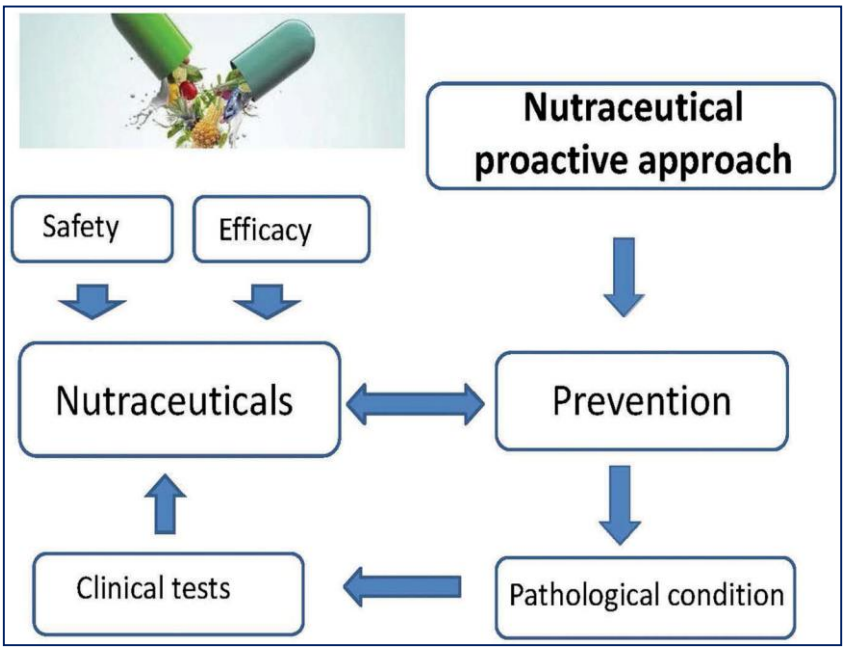


Figure 1. Possible suggested nutraceutical approach to a pathological condition.

1.2 Difference between phytochemicals nutraceuticals and drugs

The term "nutraceutical" combines the two words "nutrient" (a nutritious food or a food component) and "pharmaceutical" (drug/pharmaceutical), indicating that these products have a potential therapeutic application and should be treated similarly to drugs. The terms "phytochemical" are being used interchangeably to describe the active components of plants, but not all phytochemicals have nutraceutical value. Phytonutrients or phytochemicals that are being studied that sustain or promote health and no side effects include terpenes, carotenoids, polyphenols, anthocyanidins, limonoids, glucosinolates, and phytosterols (Balderas et al 2010). The assessment of nutraceuticals' optimal conditions of use should be complementary with safety information as well as bioavailability and bioaccessibility information, so that they can propose themselves as a powerful toolbox to be used to prevent and cure some pathologic conditions in subjects who, for example, are not eligible for conventional pharmacological therapy (Santini and Novellino. 2017). Nonetheless, there are many difference between pharmaceutical and nutraceuticals. The main challenge is the absence of a shared supra-national regulation for nutraceuticals, which would recognize their potential and possible role as therapeutic tools in some pathological conditions based on assessed safety, known mechanism of action, clinically proven efficacy in both reducing the risk of illness onset and enhancing overall well-being (Santini et al. 2018). Moreover, to obtain a marketing authorization, a drug must prove to be safe and effective for the intended use through the results of clinical trials conducted on humans. Nutraceuticals, on the other hand, do not necessarily consist of a single active component, so the expected health effect result from the synergic action of different phytochemicals present in the food matrix. Nutraceuticals often present minimal side effects compared to conventional drugs, as well as having, in many cases, greater bioavailability. They are therefore a promising tool for health management, capable of blurring the boundary between drugs and food. According to the current regulation the nutraceuticals are considered in the same category of the food supplements, notwhitstanding the different definitions. As for this situation there is a lack of shared regulation and the need to define them as a novel category (Tripathy et al. 2015).

1.3 Functional food

Functional foods are food and food derivatives that provide beneficial health effects in addition to their basic nutritional properties (Menrad, 2003). It should be a food similar in appearance to a conventional food (e.g. beverage, food matrix) consumed as part of a usual diet, which contains biologically active components with proven physiological benefits capable to give the potential of reducing the risk of chronic diseases beyond their basic nutritional functions (Food and Agricultural Organization of the United Nations (FAO), 2007).Theseformulations are obtainedbyaddingmicronutrients, like vitamins, minerals, essential amino acids, prebiotics, and probiotics. Several *in vitro*, animal models and human studies, have demonstrated that functional foods and nutraceuticals may improve postprandial hyperglycemia and adipose tissue metabolism modulating carbohydrate and lipid metabolism.Functional foods can be categorized and some examples are shown in Table 2 (Jenzer et al. 2016).

Category	***Definition***	***Examples***
Basic Food	Food or food product that naturally contains bioactive compounds	• Carrots naturally containsBeta-carotene
Processed Foods with added bioactive	Bioactive agent is added to the food during the processing	• Orange juice added with calcium • Yoghurt with increased level of probiotic • Corn flakes combined with folic acid and mineral salts
Foods enhanced to have more of bioactive	Normally Bioactive level is modified or concentrated	• Tomatoes with increased lycopene content • Eggs with increased levels of omega-3 fatty acids • Milk added with coenzyme Q10

Table 2. Selected categories of functional foods.

1.4 Impact of natural approaches in diabetes

1.4.1 Diabetes and risk factors

During the last twenty years the prevalence of diabetes has increased dramatically in many parts of the world and this disease is now a worldwide public health problem (WHO 2016).Diabetes mellitus (DM) is a metabolic disordercharacterized by hyperglycemia, dyslipidemia, insulin resistance, impaired beta-cell functioning, in which the body cannot regulate the amount of sugar in blood. This is caused by defects in insulin secretion, action or a combination of both,being(Teoh et al. 2009).

two major types of the disease:

• Type I: there is little or none insulin production, and insulin injections are needed daily.

• Type II: insulin resistance is present and glucose is unable to enter the cells to be used stored or used as energy.

Type 1 DM (formerly known as insulin-dependent DM) is an autoimmune disorder characterized by the destruction of pancreatic β-cells, where insulin injection is currently the only treatment.Type 2 DM (formerly known as insulin-independent DM) patients present insulin resistance compensated by a β cell hyper secretion of insulin (Zaccardi et al. 2016).

Risk factors, like genetic predisposition, lack of balanced diet, inappropriate and lethargic lifestyle, overweight, obesity, oxidative stress, and lack of probiotics in gut, are predisposing factors either alone or in synergy. An increasing number of epidemiological investigations show that a diet rich in foods with high content of phytochemicals, high total antioxidants, and polyphenolic compounds may be related to a lower risk of diabetes and its predisposing factors.

Many studies (Battisi et al. 2003; Victoria et al. 2009, Alokail et al. 2013) showed beneficial effect of dairy products in reducing T2DM and other associated health disorders like obesity and cardiovascular diseases.

Numerous drugs are available, such as biguanides, sulphonylurea agents, thiazolidindiones, α-glucosidase inhibitors, GLP-1 agonist, and DPP-4 inhibitors, but the clinical outcomes are often aligned with side effects, e.g. glucose intolerance, and hypoglycemia. Taking into account the reported data,it seems necessary to develop new approaches which use natural products that have health benefits, low toxicity incidence and less side effects.

Evidences indicates that antioxidant-based nutraceuticals/functional food are an optimal tools for the glycaemic control and also that the daily diet plays an important role in the prevention of T2DM.

Most medicinal plants contain micronutrients, amino acids and proteins, mucilages, essential oils, sterols and triterpenoids, saponins, carotenoids, alkaloids, flavonoids, phenolic acids, tannins, bitter principles, and coumarins. Some of these phytochemicals have been reported to have hypoglycemic activity;thisprompted to exploit in particular in my study the carotenoids, triterpenoids, and polyphenols concentrated in the food by products as analyzed in the following.

2. Food waste valorization as a source of bioactive compounds: case studies

Attention to the agro-food industry by products stems from the possibility of recovering the waste from the processing of raw materials as a source of biologically active compounds such as polyphenols, flavonoids, terpenoids, carotenoids, creatine, polysaccharides,etc.which have beneficial effects for human health(Rudra et al. 2015).

Effective utilization of food waste suitable for the formulation of nutraceuticals and or functional foodsis an emerging area of interest. As an example, the pomace resulting from the vinification has still a high content of flavonols, anthocyanins and resveratrol, that have a strong antioxidant activity. This suggests a potential source of nutraceutical with health beneficial properties (Gestuvo and Hung 2012). The whey, a by-product of the dairy industry, is recognized as a source of lactose, minerals and, above all, of bioactive peptides with interesting antioxidant, antimicrobial, antihypertensive, and immunomodulatory properties (Brandelli et al., 2015).

Considering these premises, the focus of my research has been addressed to the recovery of: A) tomatoes waste; B) peaches fruits thinning; C) olive leaves. The evaluated matrices areshown in Figure 2and described in the following.

A) **B)** **C)**

Figure 2. Food waste matrices analyzed in my research study.

2.1 Tomatoes by products: chemical composition and biological properties

Tomato (*Solanum lycopersium),* belongs tothe*Solanaceae*family, one of the most popular and extensively consumed vegetable crop worldwide. Countries insisting on the Mediterranean sea, e.g. Italy, are among the main producers of tomatoes in Europe and the waste of the industrial processing of tomatoes represent a low cost and relatively cheap source of molecules with nutraceutical potential. The chemical composition of tomato processing waste fractions was characterized by Al-Wandawi et al., since1985. During the industrial processing of tomatoes, are generated large quantities of wastes, more than 40%, and they consist of peels, seeds, fibrous parts, and pulp residues that account for 7.0–7.5%of the raw materials (Sogi et al. 2002). The most interesting components part is represented by antioxidants: vitamin C (160-240 mg/Kg), provitamin A (6-9 mg/Kg, including β-carotene), vitamin E (5-20 mg/Kg) and phenolic compounds, mainly flavonoids (5-50 mg/Kg), and phenolic acids (10-50 mg/Kg). In the peel, more correctly called epicarp, there are two compounds with antioxidant activity, belonging to the class of carotenoids: lycopene and β-carotene,which are essential for human health. This suggested the exploitation of tomato by-products in the nutraceutical and pharmaceutical industries as shown in Figure 3.

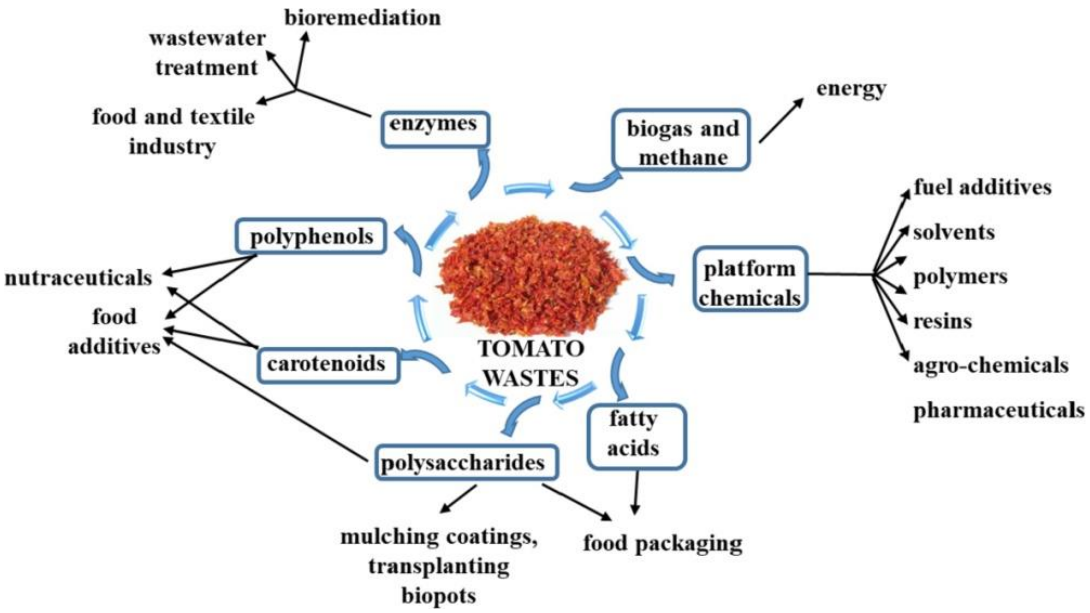


Figure 3. Tomato wastes main products and respective application.

Some authors have evaluate bioactive properties and chemical aspects of tomato waste (George et al 2004;Bose and Agrawal 2006; Luvizotto et al. 2015)highlightthatthese by-products are promising sources of bioactive constituents for the management ofdiabetes mellitus.

2.1.1 Carotenoids: human biological activity in the treatment of diabetes mellitus

Carotenoids are a class of natural antioxidants that occur in many vegetables, marine sources as colorful pigments, fruits, andtomatoes,these last are among the targets of the proposed research. Most carotenoids such as β-carotene, lycopene, lutein, zeaxanthin, astaxanthin, are lipophilic tetraterpenoids (40-carbon skeleton) and are shown in Figure 3.

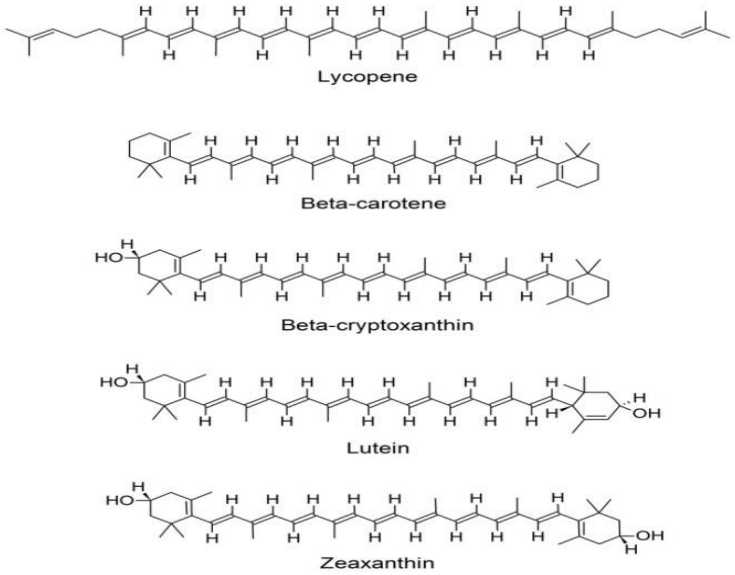
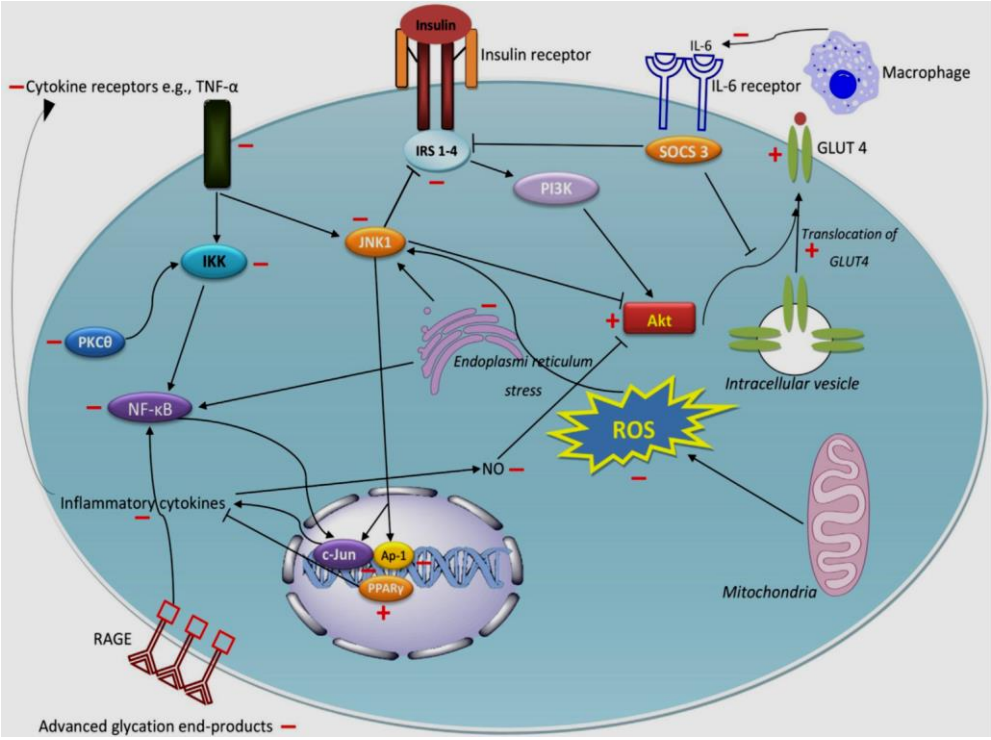


Figure 4: Chemical structure of the most preventative carotenoids compounds.

The presence of carotenoids in the diet and their role in human health has become a subject of extraordinary interest. Due to their unique structure, they protect tissues against oxidative damage by free radicals or reactive oxygen species produced as a result of the metabolic and pathological processes.

The role of carotenoids in the prevention and treatment of T2DM has been investigated in different studies. Recent advances on biological properties of carotenoids have shown that these compounds are able not only to prevent but also treat or ameliorate diabetes and its possible subsequent complications.

It has been observed that carotenoid intake has an inverse relation with HbA1c level (Suzuki et al. 2002), are inversely associated with fasting plasma glucose concentrations and insulin resistance, which implies that carotenoids may increase the insulin sensitivity(Sluijs et al. 2015). In addition, recent findings have confirmed the protective roles of carotenoids against diabetic retinopathy (Brazionis et al. 2009). Interestingly, data published seem to suggest also that PPAR-γ receptors, which have an important role in the metabolism of carbohydrates, are a target for carotenoids. In this respect, a new way to reduce the plasma sugar level via the induction of PPAR-γ expression receptor, suggesting a pathway similar to thiazolidinediones, a class of oral antidiabetic drugs used in clinical practice (Takahashi et al. 2009).Possible targets of carotenoids for their anti-diabetic effects are shown in the Scheme 1.



Scheme 1. An overview on the targets of carotenoids for their anti-diabetic effects. +, promote/activate;- inactivation (Sluijs et al 2015).

2.1.2 Lycopene and its incidence on diabetes mellitus

Lycopene,avery potent antioxidant of carotenoidsgroup,has received considerable scientific interest in recent years for its potential role in the prevention of oxidative stress-related chronic diseases.By acting as an antioxidant, it can trap reactive oxygen species (ROS), reduce oxidative stress and the danger of oxidation of cellular components, including lipids, proteins and DNA(Prabhakar and Doble 2011).A study by Rajaei et al. 2013 showed that diet containing lycopene significantly prevented glucose and insulin intolerance, decreased the hepatic glycogencontentandpancreasdamage. Moreover, lycopene notably prevented the increase of IL-1β, TNFα and CRP. *In vivo* experimental data have demonstrated the capacity of lycopene, mainly contained in tomatoes and vegetables, to improve glycaemia as well as other metabolic disorders in mice given a high-fat diet after twelve-week of oral supplementation, either as a pure compound or as tomato powder, at the same dosage (Fenni et al. 2017). Another evidence has been suggested in a study on antihyperglycemic effect of lycopene in alloxan induced diabetes in rats,whichconcluded that lycopene (5mg/Kg) can produce a significant reduction in blood glucose level and has antidiabetic activity. (Akilandeswari et al. 2015).

The work presented inthis thesis provides an important contributionby evaluating the effect of tomato waste (*Solanum lycopersicum* L.) withregardtodiabetes condition, asconfirmedby many researches indicating that the beneficial health effects are due to the presence of bioactive molecules such as carotenoids, particularly

lycopene, and polyphenolic compounds, as the flavonoids. Following the objective of maximizing the functional value of natural matrices several efforts have been made in order to maximize the accumulation of carotenoids and polyphenols in the same material.

Figure 5. Different variety of tomatoes utilized in my researchto recover the waste.

2.2 Nectarin thinning waste

Fruit thinning is suggested to be performed after fruit set and involves the removal of the small and/or damaged fruit from the tree. It is generally applied to a specific range of tree fruits, including apples, pears, plums, peaches, and nectarines.A minimum of one fruit every 5–8 cm (plums and apricots) to a maximum of one fruit every 10–15 cm (apples and pears) and 20–25 cm (peaches and nectarines) on tree branches (www.rhs.org.uk/advice/profile) are left on the plant. The thinned fruits are considered then a “waste” which should be disposed and may be an economical and environmental issue (Kulkarni andAradhya 2005). Recent research has shown that fruits from thinned trees also have a higher concentration of polyphenols beneficial for human health and nutrition.

Thinningis the most cost effective and efficient methods of recovering waste from fruits. This improves fruit quality and triggers the search for new approaches of recovery and reuse of active compounds. For these purpose, my research *in vitro*and*in vivo* aimed to study the possibility of using thinned fruits to produce novel nutraceuticals for the first time, thanks to the content of phytochemicals related to diabetes condition, particularly the presence of the phytohormone abscisic acid,which is more present in immature stage of the fruits, giving the maximumbeneficial effect in human gluco-metabolic conditions.

2.2.1 Abscisic acid:its role in plants

The isoprenoid phytohormone abscisic acid (ABA), shown in Figure 4, is considered one of the five main plant hormones. It plays important roles during many phases of the plant life cycle including seed development and dormancy. Since its discovery in the early 1960's, ABA has received considerable attention and more recently, has been consideredfor its possible medicinal use in humans. The discovery of ABA synthesis in animal cells has generated interest in the possible parallels between its role in plant and animal systems.Abscisic acid acts as a regulator of plant growth, induces expression of cell cycle inhibitors effective on DNA and protein synthesis and thereby arresting cell divisions and blocking cell cycle progression at the initial steps. Probably, this effect may be highly requested by the fruit at an immature stage when cell cycle progression can be disturbed by several environmental factors such as oxidative stress (Wang et al. 2012). Later, the production of increasing levels of protective compounds, such as the antioxidants, would make possible the fruit complete growth, so that the action of ABA is no longer required (Swiatek et al. 2002). ABA can specifically target guard cells for induction of stomatal closure, but may also produce a systemic response during periods of drought stress.

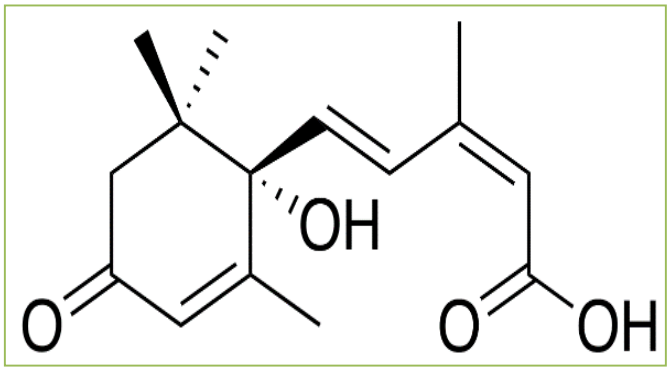


Figure 6. Chemical structure of abscisic acid.

Table 3 shows the amount of this phytohormone in a variety of fruits and vegetables.The concentration varies depending on the type of the plant but, on average, the ABA concentration is about 0.29/mg/Kg wet weight of vegetable and 0.62 mg/Kg of wet weight of fruit. Among these vegetal matrices, it is worth to note the rare vegetable Okra (*AbelmoscusEschulentus*) where for the first time in our laboratory this molecule has been identified in an amount of 7 μg/g. This research allowed to conclude, after evaluating its bioaccessibility, that Okra phytocomplex can be proposed as a novel nutraceutical to be administered in gastro-resistent capsules for glycemic control.

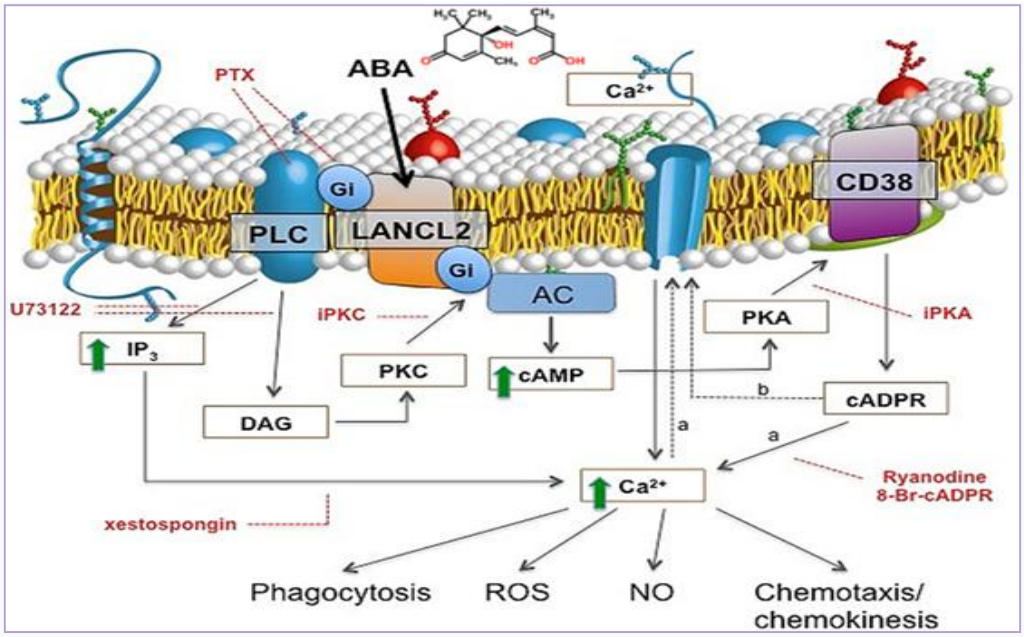
Food	Part	ABA, µg/g	Reference
Eureka lemon	Dried peel	6.5	J. Exp. Bot., 1991, 42 (241), 1083-1088.
Lisbon lemon	Dried peel	7.8	J. Exp. Bot., 1991, 42 (241), 1083-1088.
Red blush grapefruit	Dried peel	3.6	J. Exp. Bot., 1991, 42 (241), 1083-1088.
Marsh white grapefruit	Dried peel	3.7	J. Exp. Bot., 1991, 42 (241), 1083-1088.
Washington Navel Orange	Dried peel	6.4	J. Exp. Bot., 1991, 42 (241), 1083-1088.
Washington Navel Orange	Dried peel	8.3	J. Exp. Bot., 1991, 42 (241), 1083-1088.
Tall Satsuma mandarin	Dried peel	2.9	J. Exp. Bot., 1991, 42 (241), 1083-1088.
Dwarf Satsuma mandarin	Dried peel	8.3	J. Exp. Bot., 1991, 42 (241), 1083-1088.
Orange	Rind	1.1	Planta 1983, 157, 371-375
Avocado	Mesocarp	7.6	Planta 1983, 157, 371-375
Bean	Leaves	0.08	Planta 1983, 157, 371-375
Fig	Whole	0.73	Magnone, The FASEB Journal fj.15-277731.
Bilberry	Whole	0.4	Magnone, The FASEB Journal fj.15-277731.
Apricot	Whole	0.32	Magnone, The FASEB Journal fj.15-277731.
Banana	Whole	0.22	Magnone, The FASEB Journal fj.15-277731.
Potato	Whole	0.03	Magnone, The FASEB Journal fj.15-277731.
Soy milk	Whole	0.03	Magnone, The FASEB Journal fj.15-277731.
Apple	Whole	0.024	Magnone, The FASEB Journal fj.15-277731.
Olive	Whole	0.016	Magnone, The FASEB Journal fj.15-277731.

Table 2. ABA amount in fruit and vegetables.

2.2.2 Biological activity of abscisic acid in human glucose metabolism

Since several evidences suggest that ABA is involved in the regulation of glucose metabolism in humans, the importance of this molecule has prompted the development of several study which have been addressed to analyze and evaluate this phytohormone properties. The first report concerning the function of ABA in mammalian glucose homeostasis was elaborated on the basis of the structural similarity of this hormone with the thiazolidinediones, a class of insulin-sensitizing anti diabetic drugs. A unique finding in mammalian systems, however, is that the peroxisome proliferator-activated receptor PPARγ is up regulated by ABA in both in vitro and in vivo studies (Guri et al., 2007).

This result, showed no direct effect of ABA on insulin secretion, an aspect later clarified by Bruzzone et al. in a 2008 study, in which it was verified that the ABA, in nanomolar concentrations, enhances the release of insulin by both murine and human cells, using the same signaling pathway that involves cAMP, PKA, and ADP-cyclical as shown in the Scheme 2.



Scheme 2. ABA signaling pathway in human cells.

In addition, pancreatic β-cells release ABA in response to glucose, and ABA, in turn, increases and stimulates glucose-dependent and glucose-independent insulin secretion, suggesting the existence of an autocrine/feed-forward cycle (Bruzzone et al., 2008).The same authors also suggested in 2015 a possible positive feedback mechanism where GLP-1 helps to stimulate the endogenous release of ABA, which in turn stimulates further secretion of insulin. (Bruzzone et al., 2015).

The role of ABA in regulating blood glucose is shown schematically in Figure 5. It is essential to highlight that it has also been shown that ABA induces in vitro glucose uptake by adipocytes and murine myoblasts increasing translocation from intracellular vesicles to the GLUT-4 plasma membrane (Bruzzone et al., 2012).

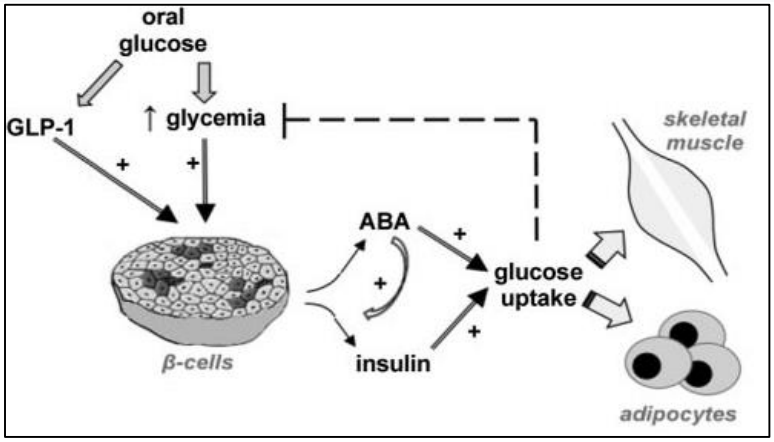


Figure 7. Schematic representation of the role of ABA in blood glucose regulation.

(Bruzzone et al. 2012).

2.3 Olive leaves

Olive tree (*Olea europaea L.*) leaves,whose obtainment is shown in Scheme 3, have been widely used in traditional remedies in European and Mediterranean Countries as extracts, herbal teas, and powder. They contain polyphelosbioactive compounds e.g. oleuropein, that have hypoglycemic properties.

Polyphenols are secondary plant metabolites widely distributed in plant tissues,andusually accumulate in the skin parts. Dietary polyphenols derive mainly from plant-based foods including fruits, vegetables, whole grains, coffee, tea, and nuts(Arts and Hollman, 2005).Over the years, research has been addressed to find out the effect of phenolic compounds for their biological activity as antioxidant and anti-inflammatory agents against different diseases (Fraga et al. 2010).Polyphenols may influence glycemia and type 2 diabetes (T2D) through different mechanisms, such as promoting the uptake of glucose in muscle and adipocytes (Leiherer et al. 2012)and therefore improving insulin sensitivity. Hyperglycemia onset starts with the elevation of free radicals in the body. On the other hand, dietary habits and diet supplementation with antioxidant are able to decrease the possible complications accompanied to oxidative stress in T2D (Osawa and Kato, 2005).

The most preventive polyphenols with took our attention as potential compounds in nutraceutical formulations are described in the following.

2.3.1 Oleuropein

Oleuropein (OLE) is the most prevalent polyphenol present in olives leaves, the agro-industrial waste matrices studied in this research thesis.

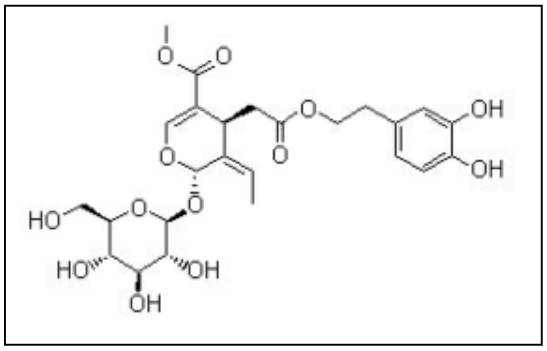


Figure 8. Chemical structure of oleuropein.

Few studies have been conducted to elucidate the mechanisms of action of OLE in management of T2DM as shown in Figure 7. One of the first proposed mechanisms is the inhibition of several enzymes involved in glucose metabolism. Oleuropein has been reported as a good antioxidant *in vitro* through chelation of Cu^{2+} and Fe^{3+} metallic ions which then catalyse free radical formation. Studies *in vivo* indicated the inhibition of enzymatic oxidation e.g. lipoxygenases (Gordon et al. 2001; Al-Azzawie and Alhamdani,2006). Besides this, interestingly in healthy subjects, the administration of 20 mg OLE significantly increased the levels of

glucagon like peptide (GLP-1) compared to placebo, contributing to attenuate the postprandial glycaemic levels (Carnevale et al. 2018). A recent in vitro study demonstrated that OLE activates the AMPK which regulates cellular energy metabolism (Hadrich et al. 2016). In particular, OLE has been reported to prevent the β-cells damage caused by oxidative stress (Rigacci and Stefani, 2016) by activating the Nuclear transcription factor (erythroid-derived-2)-like 2 (Nrf2)/Antioxidant response element (ARE) and upregulating protective enzymes, such as the thioredoxin reductase (Cavalot et al. 2006; O'Keefe and Bell. 2007;Konstantinidou V. 2009,).

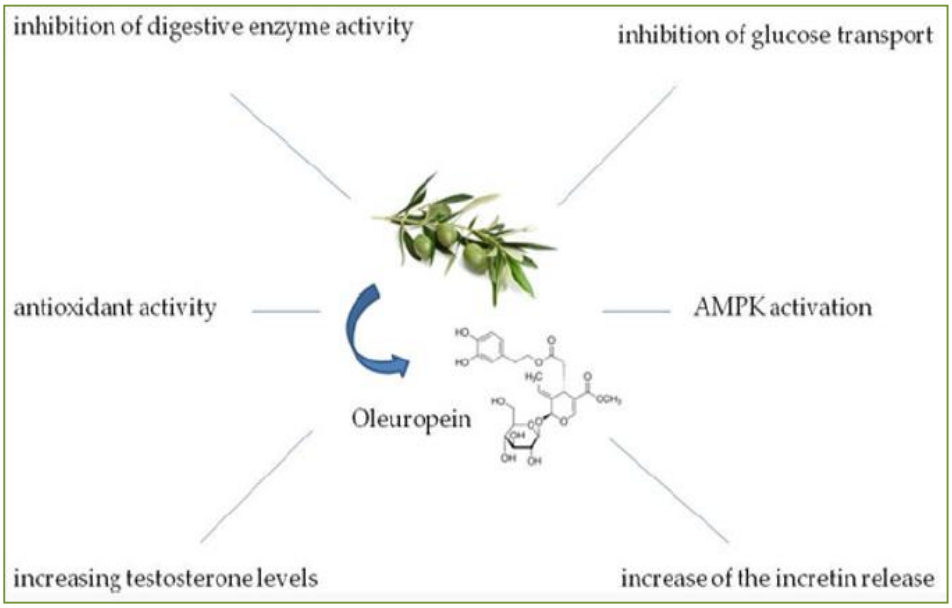
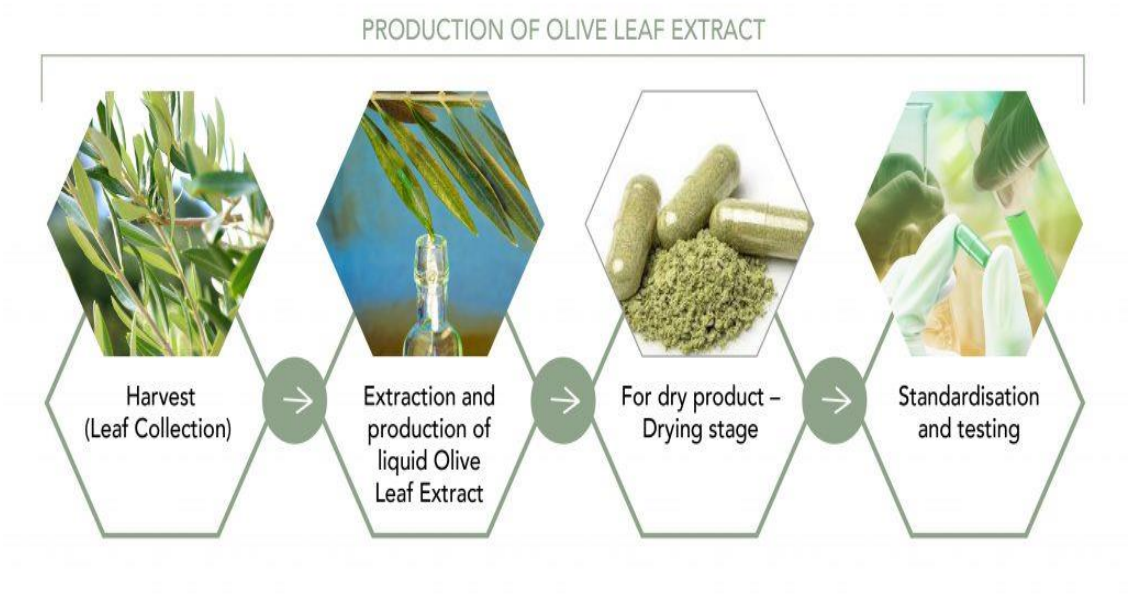


Figure 9. Main mechanisms of action for the anti-diabetic effect of OLE (Annunziata et al. 2018).

The efficacy of 500 mg oral olive leaf extract taken once daily in tablet form versus matching placebo in improving glucose homeostasis in adults with type 2 diabetes (T2DM)has been recently repoted(Arts and Hollman, 2005).

In some cases evidence have been reported that these matrices are a host of fungal contamination. Recent studies showed the presence of secondary metabolites (mycotoxins) of toxinogenicmoulds (*Aspergillus*spp) in olives leaves and fruits. Some species, in particular *Aspergillus flavus*and*Aspergillus ochraceus*are capable to produce aflatoxin B1 and ochratoxin A (OTA) (Choi et al. 2015). In this case, the oleuropein extracted from such olives can be contaminated by small quantities of these mycotoxins.This fact triggered us to speculate for novel alternative of biocontrol agents as a preliminary step before the standardization and testing the extract,with an improvement of the quality of the end products.



Scheme 3: Olive leaf extract production steps.

Chapter II

Nutraceuticals and mycotoxins contamination: probiotics as biocontrol agents for safe approach

Introduction

Nowadays the continuous growing interest of consumers for natural and safe products and the problems associated with the use of chemical pesticides and food preservative makes it necessary to search suitable protective alternatives.

The International standards and the European legislation well describe the so called*known mycotoxins* such asaflatoxins (AF), ochratoxin A (OTA), and trichothecenes, present in a variety of crops. However, information of mycotoxins in fresh fruit and vegetable are limited. Thus, in order to mitigate mycotoxin contamination, control approaches are used. One of the safe methods proposed is the Bio-preservation, defined as the use of microorganisms, as well as their metabolic products, to prevent fungal growth and improve the food shelflife (Woo& Pepe, 2018). Lactic acid bacteria (LAB) are microorganisms commonly used in numerous industrial fermentation processes (Martinez et al., 2013) and represent also a promising strategy against mycotoxins, which are resistant to washing process and temperature,to prevent spoilage of fruits and vegetables (Barbosa et al. 2015; Lay et al. 2016).The presence of mycotoxin contamination in food has adverse human health effects and nowadays has become of global interest.

To explore the possibility to use lactic bacteria as a biocontrol agent against secondary metabolites from microfungi contaminating crops, tomatoes have been chosen as a case study.Tomatoes are rich in bioactive compounds and are highly susceptible to fungal contamination due to their soft epidermis. The industrial processing of tomatoes, such as heating, pasteurization, sterilization, and storage has no significant effect in reducing these mycotoxins in the final products. It has been observed that the exposure to these toxins may damage β cells triggering the progression to diabetes onset (Larsen et al.2001). Moreover, some studies highlighted a bi-directional relationship existing between mycotoxins and gut microbiota.The gut microbiota plays a crucial role in diabetes. Recently,has been reported how the diabetogenic effects of Ochratoxin A (OTA), the most toxic product of *Aspergillus ochraceus* and *Penicillium verrucosum*, not only alter blood glucose and insulin levels in rats, but also leads to the increases in blood glucagon levels and causes pancreatic lesions (Pleadin et al.2001).

For these purpose, considering the microbiological quality assessment, my research has been conducted to evaluate the efficacy ofnaturalprotectivealternatives by using ten different strains of *Lactobacillus spp.* and *Bacillus spp.*as biocontrol agents against tenmycotoxinogenic fungi and identify-quantify the secondary metabolites produced from these strains, potentially responsible for the antifungal activity, such as organic

acids, phenolic acids, and volatile organic compounds (VOCs). This step has been developed in order to check bothquality and safety of a natural source like tomatoes, as a preliminary and important key stepbefore the nutraceutical or functional food formulation.

2. Material and Method

2.1 Material

The standards of phenolic compounds: gallic acid, chlorogenic acid, caffeic acid, syringic acid, vanillin, *p*-coumaric, hydroxybenzoic acid, vanillic acid, hydroxycinnamic acid, sinapic acid, benzoic acid, DL-3-phenyllactic acid, 1,2-dihydroxybenzene, 3,4-dihydroxyhydrocinnamic acid and DL-*p*-hydroxyphenyllacticacidwereobtained from Sigma–Aldrich (Dublin, Ireland). Phenyllactic acid (PLA) was obtained from BaChem (Weil am Rhein, Germany). Ferulic acid was purchased from MP Biomedicals, and protocatechuic acid from HWI Pharma Services (Ruelzheim, Germany). All analytes had a purity of 95 %.Liquid chromatography grade solvents, including acetonitrile (ACN), methanol, ethyl acetate and formic acid (99 %) were obtained from VWR Chemicals (Radnor, USA). Magnesium sulphate ($MgSO_4$), C18, ammonium formate and sodium chloride (NaCl) were obtained from Sigma-Aldrich. Potato dextrose broth (PDB), potato dextrose agar (PDA), de Man–Rogosa–Sharpe (MRS) broth, and MRS agar, Glucose, Malt extract broth, Yeast extract, Triptone soy broth, were obtained from Liofilchem (Teramo, Italy). Deionised water (<18 MΩ cm^{-1}) was obtained from a Milli-Q purification system (Millipore Corp., Bedford, MA, USA).

2.2 Fungi and bacteria isolation

*Penicillium verrucosum*VTT D-01847 was obtained from the VTT Culture Collection (Finland), *Botrytis cinera* (CECT 20973) was obtained from the Spanish Type Culture Collection (Spain), *Aspergillus flavus* ITEM 8111, Fusarium strains (*Fusarium proliferatum* ITEM 12072), *Fusarium graminearum*ITEM 126, *F. verticillioides* ITEM 12044, *F. sporotrichioides*ITEM 12168, *F. langsethiae*ITEM 11031, *F. poae*ITEM 9151), *Alternariaalternata*(ITEM 8122) were obtained from the Agro-Food Microbial Culture Collection (Italy).

2.3 Microorganism culture preparation

Based on their specific growth medium, the studied bacteriaas biocontrol agents were divided into two groups and given a code as shown in Table 1.

	Name	Code	Medium	Temperature (°C)	Group
1	*Lactobacillus plantarum*	PP155	MRS	30	1
2	*Lactobacillus plantarum*	PA850	MRS	30	
3	*Lactobacillus plantarum*	MP1691	MRS	30	
4	*Lactobacillus brevis*	PCH 375	MRS	30	
5	*Lactobacillus plantarum*	TR710	MRS	30	
6	*Bacillus licheniformis*	BL20	NB	37	2
7	*Bacillus megaterium*	BM44	NB	30	
8	*Bacillus subtilis*	BS499	NB	30	
9	*Bacillus amyloliquefaciens*	BA493	NB	37	
10	*Bacillus thuringiensis*	BT197	NB	30	

Table 1. Biocontrol agents used and their conditions of growth.

Medium ingredients

De Man, Rogosa and Sharpe (MRS broth):(meat peptone 10.0 gL^{-1}; dextrose 20.0 gL^{-1}; yeast extract 5.0 gL^{-1}; beef extract 10.0 gL^{-1}; disodium phosphate 2.0 gL^{-1}; sodium acetate 5.0 gL^{-1}; ammonium citrate 2.0 gL^{-1}; magnesium sulfate 0.1 gL^{-1}; manganese sulphate 0.05 gL^{-1}; Tween 80 1.0 gL^{-1}). Preparation note:26 ghave been solubilizedin 1 L of distilled water.

Nutrient broth (NB): meat peptone 10.0 gL^{-1}; beef extract 10.0 gL^{-1}; sodium chloride 5.0 gL^{-1}; Preparation note: 28 g have been solubilized in 1 L of distilled water.

2.4 Growth of strains for preparation of cell-free supernatant

The bacteria were cultivated in corresponding medium (MRS or Nutrient Broth) at 25-37 ºC until the exponential phase growth (12 h). Then, the strains were inoculated at a concentration of 10^7 CFU mL^{-1} in 200 mL of each medium and incubated at 37 ºC for 72 h. After fermentation, LAB were separated by centrifugation at 3200 g for 10 min. Cell-free supernatants (CFS) were stored at –80 ºC for 24 h before lyophilisation (FreeZone 2.5 L, Labconco, Kansas City, MO, USA) and then stored at –19 ºC.

2.5 Qualitative assay *in vitro* of antifungal activity in solid medium

2.5.1 Diffusion agar test

The effect of the bacteria on different fungal strains growth was evaluated using two qualitative methods. On one hand, the diffusion agar method was used to study the effect of fermented CFS against fungi. Potatoes dextrose agar (PDA) plates were inoculated with fungal spores using sterile cotton swabs. Then, the wells were made using sterile pipette tips, and each well was loaded with 100 μL of lyophilized CFS and suspended in Milli-Q water to a concentration of 250 mg mL^{-1}. A well with lyophilized of medium was included as the negative control. Afterward, the plates were incubated at 25 °C for 48 h. Finally, the inhibition halo diameter was measured. Halos larger than 8 mm were considered positive for antifungal activity (Varsha, Priya, Devendra, &Nampoothiri, 2014).

2.5.2 Dual Culture

The selection of biological control agents usually starts with an in vitro screening of a collection of strains against selected pathogens by a dual culture assay. Tenmicrofungistrainswere assigned a code as listed below and evaluated.

Name	**Code**
Alternaria alternate	646
Botrytis cinerea	181
Penicillium verrucosum	49
Aspergillus flavus	146
Fusarium proliferatum	880
Fusarium verticilloides	883
Fusarium sporotrichoides	846
Fusarium langsethiiae	850
Fusarium poae	902
Fusarium graminearum	838

2.6 Microplate quantitative assay of antifungal activity

2.6.1 Minimum inhibitory concentration (MIC)/minimum fungicidal concentration(MFC)

The assayswere performed as described by Luz et al. (2019) with some modifications. A volume of 100 µL of fermented CFS at a final concentration ranging from 0.1 to 100 $gL^{-1}$was added to 96-well sterile microplates. Next, the microwells were inoculated with 100 µLtaken from a $5\times10^{4}mL^{-1}$spores suspension in PDB of the toxigenic fungi. The positive control consisted of inoculated PDB medium with non-fermented CFS (100 g L^{-1}), and the negative control was a non-inoculated PDB medium without any treatment. Inoculated microplates were incubated at 25 °C for 72 h. The minimum inhibitory concentration is defined as the lowest concentration of the fermented CFS at which the fungi did not show any visible growth. Four replicates of each assay were done.

After determining the MIC, the concentration corresponding to the MIC and higher concentrations were sub-cultured on PDA plates for the determination of the minimum fungicidal concentration. After incubation of the plates at 25 ºC for 72 h, the MFC results were defined as the lowest extract concentration in which a visible growth of the subculture was prevented. The *in vitro* antifungal activity data were used to select LAB/Bacillus with a higher relative antifungal activity.

2.6.2 Identification of organic and phenolic acids in CFS

For the analysis of organic acids, lyophilised CFS was diluted in water and injected into the high-performance liquid chromatography (HPLC) system (Agilent 1100 Series HPLC System, Agilent Technologies, Palo Alto, CA, USA), equipped with a quaternary pump and a diode array detector, using a 20 µL sample injection loop (Khosravi et al., 2015). The analytical separation was achieved with a Spherisorb S5 ODS2 (4.6 mm × 250 mm, 5 µm) reverse-phase column (Waters Corp., Milford, MA, USA) using an isocratic mobile phase of acidified water (pH 2.1) at a flow rate of 0.6 mL min^{-1} for 25 min. The chromatogram was monitored at 210 nm. Data were acquired by the HP-CORE ChemStation system (Agilent Technologies, Santa Clara, CA, USA).

For the identification of the phenolic acids, the CFS was purified using the QuEChERS method to remove possible interferents before the chromatographic analysis (Brosnan, Coffey, Arendt, &Furey, 2014). Ten mL of fermented CFS were extracted with 10 mL of ethyl acetate, 1 % formic acid, 4 g $MgSO_4$ and 1 g NaCl, then vortexed for 1 min. The extract was then centrifuged. The supernatant was combined with 150 mg C18resin and 900 mg $MgSO_4$ and vortexed for 1 min. The extract was centrifuged again, and the supernatant was evaporated under Nitrogen flow. Immediately before the chromatographic analysis, the purified extract was resuspended in 1 mL of a mixture H_2O/ACN (90:10 v/v).

The HPLC system used for the chromatographic determination was an Agilent 1200 (Agilent Technologies, Santa Clara) equipped with a vacuum degasser, autosampler and binary pump. The column was a Gemini C18 (50 mm × 2 mm, 100 Å, 3-μm particle size; Phenomenex).The mobile phases consisted of water as solvent A, ACN as solvent B, both acidified (0.1 % formic acid), with gradient elution, as follows: 0 min, 5 % B; 30 min 95 % B; 35 min, 5 % B. The column was equilibrated for 3 min before every analysis. The flow rate was 0.3 mL min^{-1}, and 20 μL of sample was injected. Mass spectrometry (MS) analysis was conducted using a Q-TOF-MS (6540 Agilent Ultra High Definition Accurate Mass), equipped with an Agilent Dual Jet Stream electrospray ionisation (Dual AJS ESI) interface in negative ionisation mode under the following conditions: drying gas flow (N_2), 8.0 L min^{-1}; nebuliser pressure, 30 psig; gas drying temperature, 350°C; capillary voltage, 3.5 kV; fragmentor voltage, 175 V; scan range, *m/z* 20–380. Targeted MS/MS experiments were carried out using collision energy values of 10, 20 and 40 eV.

2.6.3 Analysis of VOCs of CFS

Lyophilized CFS (200 mg) was mixed with 2 mL of water and placed in a 10-mL glass vial. VOCs were identified by gas chromatography with a single quadrupole mass spectrometer detector (GC/MS) analysis. Prior to analysis, samples were incubated in a water bath at 55°C for 45 min, while being gently stirred with a rod. VOCs were extracted from the vial headspace by solid-phase microextraction (SPME). The GC system was equipped with an HP-5MS (30m × 0.25 mm, 0.25 μm 5 % diphenyl/95 % dimethylpolysiloxane) capillary column (J&W Scientific, Folsom, CA, USA). The oven was programmed to start at 40 °C (held for 2 min) and to ramp up to 160 °C at 6 °C min^{-1}, then increase to 260 °C at 10 °C min^{-1} (held for 4 min). Helium (99.999 %) was used as the carrier gas, and the flow rate was 1 mL min^{-1}. The flow was transferred from the column into an Agilent 5973 MS detector (Agilent Technologies, Palo Alto). The ion source temperature was set at 230°C, the ionizing electron energy was 70 eV, and the mass range was 40–450 Da in full scan acquisition mode. Compounds were identified using the NIST Atomic Spectra Database version 1.6 (Gaithersburg, MD, USA), considering spectra with 95 % of similarity. Results were expressed as a percentage of the VOC by dividing the area of each peak by the total area of the chromatogram peaks (Guarrasi et al., 2017). The analysis was carried outin triplicate.

2.7 *Lactobacillus plantarum PP155 and Lactobacillus plantarum 850* as a tomato bio-preservative

To evaluate the antifungal activity using CFS fermented by *L. Plantarum PP155* and*L. Plantarum* 850, tomatoes were spiked with *A. flavus* ISPA 8111(aflatoxin B_1 producer) and *P. expansum*CECT 2278. The fruits were divided into tree batches, twenty were treated with sterile MRS broth (control), twenty with CFS fermented by *L. Plantarum* PP155, and twenty with CFS fermented by *L. Plantarum* 850. First, the tomatoes were sanitized with 70 % ethanol and then washed with sterile water. A wound was made using a sterile

pipette tip. One mL of a spore solution containing 10^4 spores mL^{-1} of fungi was sprayed on the tomatoes and dried for 1 h in a laminar flow cabinet (Telstar MH 100, Terrassa, Spain). Finally, the tomatoes were treated with 10 mL of CFS at a final concentration of 12.5 g lyophilized CFS Kg^{-1} of tomato, dried and stored in a sanitized plastic box at room temperature for 9 d. At the end of storage, ten tomatoes from each treatment were separated and frozen for the determination of mycotoxins, and ten were examined for viable spores. All experiments were repeated three times.

2.8 Statistical analysis

Data were statistically evaluated using the InfoStatsoftware version 2008. The differences between the groups were analyzed by one-way ANOVA, followed by the Tukey HSD post hoc test for multiple comparisons. The significance level was set at $p \leq 0.01$.

3.Results

3.1 *Dual culture*

Several CFS of LAB and *Bacillus* showed antifungal activity against toxigenic fungi by the overlay method and in the solid medium diffusion agar test. In particular,the analysis of the activity and the percentage of mould inhibition range from 16.2% ±0.1 to 50.4% ± 0.1 as shown in Table 1. This result is most evident against the mycotoxigenic fungi belonging to *Fusarium*spp. (*F. sporotrichoide, F. graminearum and F. verticillioides*) and *Botrytis cinerea*, while a slight activity was observedfor*Aspergillus* and *Penicillium.*

The inhibition percentage has been calculatedusing the formula:

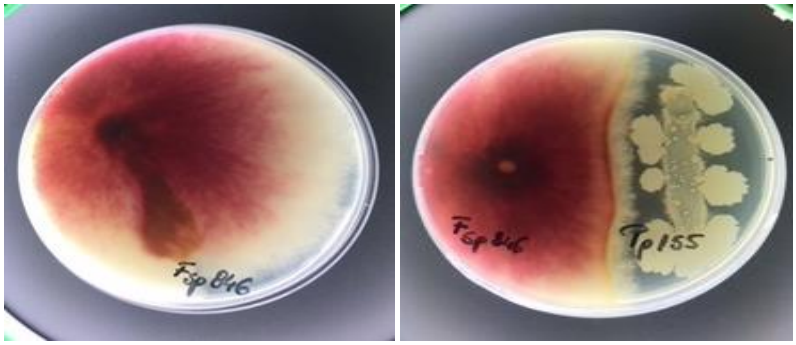
(Dmcrl-Dmstrain)/Dmcrl- 0.5 = % inhibition

whereDmcrl is the Diameter of control (cm), Dm strain is the-Diameter of strain (cm), 0.5 is the Diameter of fungi spot in the culture plate on the first day.

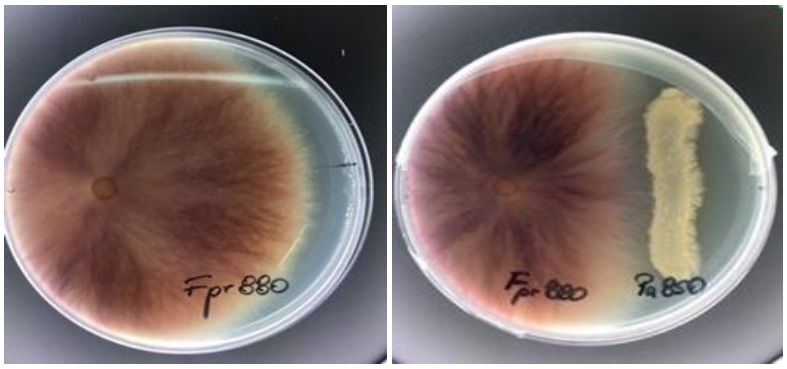
Pathogen	**F.sp846**	**F.883**	**F.pr 880**	**F.po902**	**F.ve838**	**F.la 850**	**P.ve 49**	**B.ci 181**	**A.fl 146**	**A.al 646**
Diameter plate control (cm)	8.5	6.5	7.4	6.6	5	5.3	1.6	7.7	7.5	5.5
Biocontrol										
PP 155	45.2± 0.1	50.5± 0.1	48.2 ±0.1	48.2 ±0.1	40.3±0.2	46 ± 0.2	19±0.2	38.3± 0.1	ND	34.3±0.1
PA850	50.4± 0.1	40.3± 0.1	37.6 ±0.3	20.3 ±0.1	21.2±0.1	16.6± 0.1	ND	25.3 ± 0.3	ND	22.2± 0.1
MP1691	45.1±0.2	40.3 ±0.2	50.1 ±0.3	40.2 ±0.1	39.2±0.1	40.2±0.2	20±0.2	43.6±0.4	ND	20.3 ±0.3
PCH 375	18.2 ± 0.2	17.4± 0.2	19.2 ±0.2	28.2 ±0.1	26.3±0.1	25.1 ±0.2	ND	20.2 ± 0.1	ND	19.3 ± 0.3
TR71	16.5 ± 0.1	15.4±0.1	16.6 ±0.1	18.7 ±0.2	16.2±0.2	19.4 ±0.2	ND	16.5 ± 0.4	ND	17.8 ± 0.2
BL 20	16.3 ± 0.2	17.5±0.2	18.7 ±0.2	16.9 ±0.1	ND	16.4 ±0.1	ND	16.2 ± 0.1	ND	18.7 ± 0.1
BM 44	16.2 ± 0.1	19.2 ±0.1	18.5 ±0.1	17.4 ±0.3	ND	18.3 ±0.2	ND	20.2 ± 0.3	ND	16.3 ± 0.3
BS 499	25.8 ± 0.3	20.2 ±0.3	21.2 ±0.3	25.2 ±0.2	ND	18.3 ±0.1	ND	21.5 ± 0.2	ND	19.9 ±0.2
BA493	25.3 ± 0.2	19.2 ±0.2	22.8 ±0.2	22.2 ±0.2	20.2±0.1	16.2 ±0.2	ND	33.2* ± 0.1	ND	20.3 ± 0.2
BT197	17.2 ± 0.1	19.9 ±0.1	18.2 ±0.1	16.4 ±0.3	ND	17.3 ±0.2	ND	21.2 ± 0.3	ND	16.3 ± 0.1

Table 1. Antifungal activity of isolated bacteria against *Penicillium, Aspergillus, Fusarium* and *Alternaria* species by overlay assay. Values are expressed as % of inhibition after 7 days ± standard deviation. ND- Not detected.

A)



B)



C)

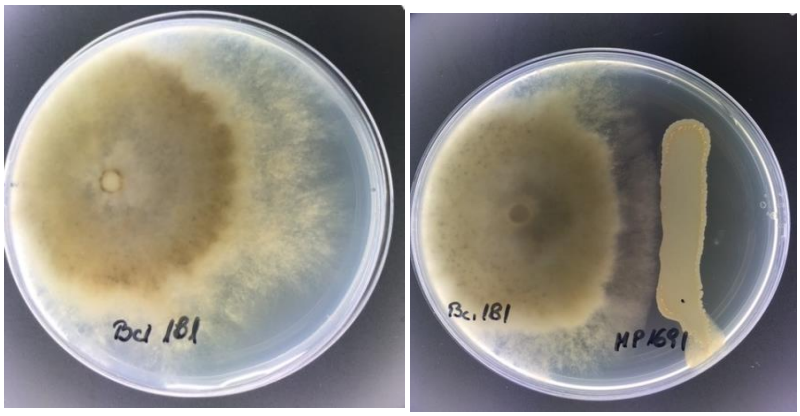


Figure 2. Examples of the antifungal capacity determined by overlay test compared with control of: A) *Lactobacillus plantarum PP155* against *Fusarium sporotrichioides (846) ITEM 12168; B) Lactobacillus plantarum PA 850* against*Fusariumgraminearum (880)* ITEM 126; C) *Lactobacillus plantarum MP1691* against *Botrytis Cinera (181)* (CECT 20973).

3.2 Agar diffusion test

Among the ten strains evaluated, the highest antifungal activity was observed in cell-free supernatants (CFS) of the *Lactobacillus plantarum* 850 and *Lactobacillus brevis PCH 375*, which inhibited the growth of tested fungi genera in solid medium as shown in Table 2 and Figure 3, respectively. Only halos larger than 8 mmhave been considered as positive result.

	Biocontrol Agents										
Target fungus	**PP 155**	**PA 850**	**BT 197**	**PCH 375**	**PA 2**	**BL 20**	**BM 44**	**BS 499**	**BA 493**	**TR 71**	**MP 1691**
Alternaria alternate	++	-	-	++	-	-	-	-	+	+++	-
Botrytis cinerea	++	-	-	-	-	-	-	-	-	+++	+
Penicillium verrucosum	-	-	-	+++	-	-	-	-	-	+++	-
Aspergillus flavus	-	-	-	+++	-	-	-	-	-	-	-
Fusarium proliferatum		-	-	-	-	-	-	-	-	++	-
Fusarium verticilloides	-	-	-	+++	-	+	-	-	-	+	-
Fusarium sporotrichoides	+++	-	-	-	-	-	-	-	-	++	+
Fusarium langsethiae	++	-	-	+++	-	-	-	-	-	++	-
Fusarium poae	-	-	-	+++	-	-	-	-	+++	+	-
Fusarium graminearum	-	-	-	+++	-	-	-	-	+++	-	-

Table2. Antifungal activity of CFS against *Penicillium, Aspergillus, Fusarium Botrytis* and *Alternaria* species by diffusion agar method. –non detected activity; + less activity; ++ middle activity; +++ strong activity.

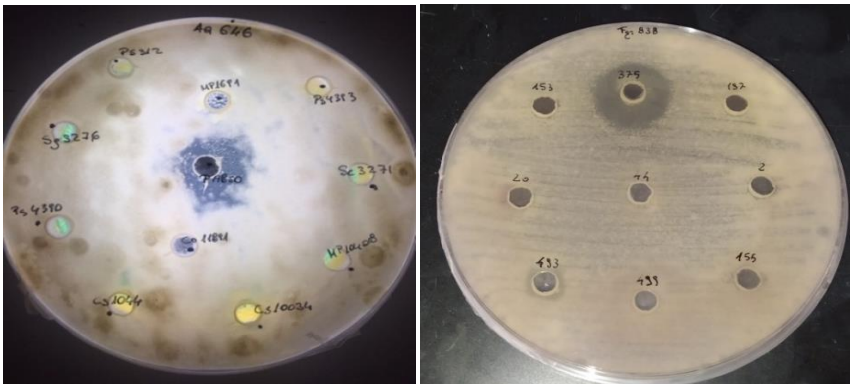


Figure 3. A) *Lactobacillus plantarum PA850* against *Alternaria alternata*ITEM 8122, B) *Lactobacillus brevis PCH 375* against *Fusarium graminearum*ITEM 126.

3.3 MIC (Minimal Inhibitor Concentration)/ MFC (Minimal Fungicide Concentration)

In order to quantify the potential antifungal activity of CFS, the MIC and MFC values were determined and the results are shown in Table 3. The aim was to find stains effective in low levels of MIC and MFC. The MIC and MFC values of CFS on *Fusarium* spp., *Penicillium* spp,*Alternaria*spp,*Aspergillus*sspp,were in the range 62.5-250 g L^{-1}. The *Fusarium* genus was the most sensitive to the compounds present in the fermented CFS, presenting the lowest average MIC and MFC values.

Fungi target	**Antifungal activity**					
	Bacteria					
	PCH 375		**PP 155**		**PA850**	
	MIC	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**
***Fusarium graminearum* ITEM 6415**	125	250	125	250	nd	nd
***Fusarium sporotrichioides* ITEM 12168**	Nd	Nd	62.5	250	nd	nd
***Fusarium langsethiae* ITEM 11031**	125	250	125	250	125	250
***Fusarium poae* ITEM 9151**	125	250	nd	nd	125	250
***Fusarium verticillioides* ITEM 12043**	125	250	nd	nd	125	nd
***Penicillium verrucosum* CECT 2913**	Nd	Nd	nd	nd	125	250
***Alternaria alternate* ITEM 8121**	Nd	Nd	125	250	125	250
***Aspergillus flavus* ITEM 8111**	125	250	nd	nd	nd	nd
***Botrytis cinera* CECT 20973**	62.5	125	nd	nd	125	250
***Fusarium proliferatum* ITEM 12072**	Nd	Nd	125	250	125	250

Table 3. Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) for lyophilized CFS (mg mL^{-1}) fermented by bacteria against: a) *Penicillium*, b) *Aspergillus*, c) *Fusarium* and *Alternaria* species. The result are expressed as (g L^{-1}).

3.4 Identification of antifungal compounds in CFS

According to the literature, the antifungal activity of the bacteria as biocontrol is not only due to a type of compound but also depends on the synergistic action between all metabolic substances present in the fermented media (Crowley et al., 2013). A total of four organic acids were determined, namely, lactic acid, acetic acid, succinic acid, and malic acid. According to our results as shown below in Table 4a), all isolated stains produced lactic acid and acetic acid with concentration in the range24–282± 0.64g $Kg^{-1}$and 21–77 ± 0.32 g Kg^{-1}, respectively. The LAB with the highest lactic acid production were: *L.Plantarum* PP155 (282.31 ± 0.84 g Kg^{-1}), *L. Plantarum PA*850 (227.74 ± 0.29 g Kg^{-1}), *L. Plantarum MP1691 (*143.83 ± 0.45 g Kg^{-1}). These values could explain the fact that high antifungal activity has been detectedfrom these strains. The production of succinic acid (14–45 g Kg^{-1}) and malic acid (6–13 g Kg^{-1}) was determined only in five and six CFS, respectively. In addition, these two acids had a lower average concentration than lactic acid and acetic acid. The phenolic acids detected in fermented CFS are listed in Table 4b. Among the eighteen targeted compounds, six phenolic acids were detected and quantified in CFS (benzoic acid, 1-2-dihydroxybenzene,DL-3-phenyllactic acid (DLA),p-coumaric acid,3-4-dihydroxyhydrocinnamic,vanillic acid). These compounds have been reported as antifungal agents produced by LAB (Omedi et al., 2019). Like the organic acid production, the phenolic acids data correlated with the observed antifungal activity of CFS. A synergism between lactic acid and acetic acid has been documented (Dagnas et al., 2015), as well as between these and DLA in the potential antifungal effect (Lavermicocca et al., 2003). A total of fiftyone VOCs have been identified in the lyophilised CFS fermented by LAB as shown in Table 4b). The compounds were classified into six groups according to their chemical class, namely alcohols, aldehydes, acids, ketones, pyrazines, and others.Our results regarding the inhibition of fungal growth by VOCs produced by bacteria are in agreement with recent reported data (Morita et al, 2019).

Organic acids	Lactic Acid Bateria									
	PP155	**PA850**	**PCH375**	**MP1691**	**TR71**	**BL20**	**BA499**	**BS 493**	**PA2**	**BM 44**
Lactic acid	282.31 ± 0.84 ^{e}	227.74 ± 0.29^{d}	160.10 ± 0.46^{bc}	143.83 ± 0.45^{b}	125.10 ± 0.12^{b}	143.68 ± 1.06^{b}	24.04 ± 0.08^{a}	200.26 ± 0.33^{cd}	194.46 ± 0.57^{cd}	34.04 ± 0.08^{a}
Acetic acid	42.53 ± 0.49^{cd}	76.93 ± 0.78^{f}	20.77 ± 0.28^{a}	29.90 ± 0.35^{ab}	30.50 ± 0.49^{b}	37.49 ± 0.78^{bc}	52.02 ± 1.41^{d}	66.14 ± 1.49^{e}	43.18 ± 0.49^{cd}	52.02 ± 1.41^{d}
Succinic acid	32.31 ± 1.44^{b}	45.46 ± 1.76^{c}	28.17 ± 0.56^{b}	31.97 ± 0.48^{b}	nd	nd	nd	nd	13.77 ± 0.40^{a}	nd
Malic acid	9.83 ± 1.81^{ab}	9.17 ± 0.74^{ab}	5.75 ± 0.20^{a}	Nd	6.85 ± 0.26^{a}	nd	13.40 ± 2.15^{b}	Nd	8.03 ± 0.34^{a}	19.30 ± 2.18^{b}

Table 4a). Identification and quantification of organic acids (g kg^{-1})produced by LAB in CFS. The results are expressed as mean ± standard deviation. Statistically significant differences for each fermentation are indicated with different letters($p < 0.01$).

Microorganims	Phenolic acids (mg/mL)					
	Benzoic acid	**DL-3-Phenyllactic acid**	**1-2-Dihydroxy benzene**	**p-Coumaric acid**	**3-4-Dihydroxyhydro Cinnamic**	**Vanillin acid**
PP 155	0.060±0.006	0.184 ± 0.003	0.044± 0.002	nd	Nd	Nd
PA 850	0.062±0.001	0.297 ± 0.002	Nd	nd	0.022 ± 0.006	Nd
PCH 375	0.023±0.001	Nd	0.040 ± 0.001	nd	Nd	Nd
TR71	0.015 ± 0.003	0.844 ± 0.006	Nd	0.002 ± 0.001	Nd	Nd
MP 1691	0.044 ± 0.001	Nd	Nd	nd	Nd	Nd
BA 493	0.004 ± 0.002	Nd	0.023 ± 0.002	nd	Nd	0.011 ± 0.001
BS 499	0.051± 0.001	0.377 ± 0.002	0.045± 0.001	0.012 ± 0.001	0.029 ± 0.004	0.023± 0.001
BM 44	0.015± 0.002	Nd	Nd	nd	Nd	Nd
PA 2	0.021± 0.003	Nd	Nd	nd	Nd	Nd
BL 20	0.019± 0.002	0.257 ± 0.005	0.015± 0.004	0.002 ± 0.004	0.008± 0.002	Nd

Table 4b). Identification and quantification of phenolic acid (mg mL^{-1}) produced by LAB in CFS.

VOC	PP155	PA850	PCH375	MP1691	TR71	PA2	BL20	BS493	BA499	BM44
ALCOHOLS	4.92± 0.01^{g}	4.84± 0.03^{g}	2.37 ± 0.07^{d}	1.89 ±0.01^{c}	3.34 ± 0.01^{f}	2.75 ± 0.02^{e}	0.72 ± 0.01^{b}	0.51 ± 0.01^{a}	0.48 ± 0.01^{a}	1.09 ± 0.01^{c}
Ethanol	0.63 ± 0.01	nd	1.54 ± 0.04	1.32 ± 0.01	0.7 ± 0.01	nd	Nd	nd	Nd	1.02 ± 0.01
3-methyl-1-butanol	nd	0.77 ± 0.01	nd	1.24 ± 0.01	nd	nd	Nd	nd	Nd	1.04 ± 0.01
3-methylacetate-1-butanol	nd	nd	nd	0.25 ± 0.01	nd	nd	Nd	nd	Nd	0.15 ± 0.01
2-ethyl-1- hexanol	4.29 ± 0.01	1.12 ± 0.01	0.08 ± 0.01	1.72 ± 0.02	1.95 ± 0.01	2.56 ± 0.02	0.53 ± 0.01	0.51 ± 0.01	0.34 ± 0.01	1.02 ± 0.02
2-nonanol	nd	nd	0.76 ± 0.03	0.33 ± 0.01	0.7 ± 0.01	nd	Nd	nd	0.15 ± 0.01	0.23 ± 0.01
2-undecanol	nd	nd	nd	Nd	nd	0.19 ± 0.01	0.19 ± 0.01	nd	Nd	nd
ALDHEYDES	11.89±0.04^{e}	7.67 ± 0.03^{d}	17.86 ± 0.5^{g}	11.93 ± 0.04^{e}	14.21 ± 0.05^{f}	6.88 ± 0.07^{c}	6.04 ± 0.08^{b}	3.97 ± 0.03^{a}	4.44 ± 0.04^{a}	8.93 ± 0.04^{e}
exanal	nd	nd	nd	Nd	nd	nd	nd	nd	Nd	nd
3-methyl butanal	0.67 ± 0.01	1.21±0.01	1.34 ± 0.04	1.45 ± 0.01	0.83 ± 0.01	0.97 ± 0.02	0.22 ± 0.01	0.12 ± 0.01	0.29 ± 0.01	1.45 ± 0.01
2-methyl butanal	0.35 ± 0.01	0.48 ± 0.01	0.57 ± 0.03	0.55 ± 0.01	0.41 ± 0.01	0.54 ± 0.01	0.2 ± 0.01	0.12 ± 0.01	0.19 ± 0.01	0.2 ± 0.01
3-methyl-2-hexanal	nd	nd	1 ± 0.03	0.15 ± 0.01	1 ± 0.01	0.17 ± 0.01	nd	nd	0.58 ± 0.01	nd
2- ethyl- hexanal	0.67 ± 0.01	nd	nd	0.14 ± 0.01	nd	0.37 ± 0.01	nd	nd	0.14 ± 0.01	nd
benzaldehyde	4.58 ± 0.01	2.57 ± 0.01	6.41 ± 0.16	3.82 ± 0.02	4.96 ± 0.01	2.12 ± 0.04	2.32 ± 0.03	1.6 ± 0.02	1.63 ± 0.02	1.32 ± 0.03
Benzene acetaldehyde	4.13 ± 0.03	2.19 ± 0.02	6.35 ± 0.19	4.2 ± 0.02	4.94 ± 0.02	2.37 ± 0.01	2.55 ± 0.03	1.46 ± 0.01	1.41 ± 0.01	1.55 ± 0.03
methional	0.95 ± 0.01	0.67 ± 0.01	1.61 ± 0.05	1.2 ± 0.01	1.47 ± 0.01	nd	nd	0.43 ± 0.01	Nd	nd
nonanal	0.57 ± 0.01	0.57 ± 0.01	0.62 ± 0.03	0.46 ± 0.01	0.63 ± 0.01	0.37 ± 0.01	0.78 ± 0.01	0.27 ± 0.01	0.22 ± 0.01	0.68 ± 0.01
ACIDS	9.45 ± 0.01^{a}	11.12 ± 0.04^{b}	18.75 ± 0.46^{f}	13.36 ± 0.02^{d}	18.14 ± 0.09^{f}	20.81 ± 0.22^{g}	13.96 ± 0.13^{e}	12.71 ± 0.09^{c}	9.2 ± 0.08^{a}	9.96 ± 0.13^{e}
acetic acid	6.5 ± 0.01	7.97 ± 0.01	11.69 ± 0.29	9.45 ± 0.01	11.53 ± 0.04	14.25 ± 0.2	9.78 ± 0.06	8.22 ± 0.04	7.31 ± 0.07	9.78 ± 0.06
3-methyl-butanoic acid	1.66 ± 0.01	1.73 ± 0.03	3.4 ± 0.07	2.15 ± 0.01	3.02 ± 0.04	3.36 ± 0.02	1.6 ± 0.02	2.09 ± 0.04	1.13 ± 0.01	1.6 ± 0.02
2-methyl-butanoic acid	1.29 ± 0.01	1.23 ± 0.01	2.37 ± 0.06	1.56 ± 0.01	2.2 ± 0.01	2.27 ± 0.01	1.2 ± 0.02	1.51 ± 0.01	0.78 ± 0.01	1.1 ± 0.02
octanoic acid	nd	0.2 ± 0.01	1.3 ± 0.04	0.22 ± 0.01	1.4 ± 0.01	0.47 ± 0.01	1.4 ± 0.05	0.91 ± 0.01	Nd	1.4 ± 0.05
2-methyl propanoic acid	nd	nd	nd	Nd	nd	0.48 ± 0.01	nd	nd	Nd	nd
KETONES	nd	0.16 ± 0.01^{a}	nd	Nd	0.4 ± 0.01^{b}	1.51 ± 0.02^{d}	1.29 ± 0.01^{c}	2.44 ± 0.04^{e}	0.11 ± 0.01^{a}	1.29 ± 0.01^{c}
2-heptanone	nd	nd	nd	Nd	0.4 ± 0.01	0.98 ± 0.01	0.65 ± 0.01	1.41 ± 0.03	Nd	0.65 ± 0.01
acetophenone	nd	nd	nd	Nd	nd	nd	nd	nd	Nd	nd
2-undecanone	nd	0.16 ± 0.01	nd	Nd	nd	0.54 ± 0.01	0.65 ± 0.01	1.04 ± 0.01	0.11 ± 0.01	0.65 ± 0.01
PYRAZINES	50.31 ± 0.04^{c}	64.61 ± 0.17^{e}	39.14 ± 3.35^{b}	47.79 ± 0.11^{c}	36.47 ± 0.24^{b}	53.55 ± 0.29^{d}	62.04 ± 0.3^{e}	70.77 ± 0.69^{f}	74.01 ± 0.77^{f}	62.04 ± 0.3^{e}
methyl-pyrazine	1.79 ± 0.01	3.06 ± 0.02	2.73 ± 0.08	1.5 ± 0.02	2.9 ± 0.03	2.02 ± 0.02	2.18 ± 0.02	1.75 ± 0.01	1.66 ± 0.01	2.18 ± 0.02
2,5-dimethyl pyrazine	5.61 ± 0.01	6.12 ± 0.01	10.73 ± 0.27	3.51 ± 0.01	13.39 ± 0.07	3.8 ± 0.02	4.53 ± 0.04	3.17 ± 0.05	2.98 ± 0.01	4.53 ± 0.04

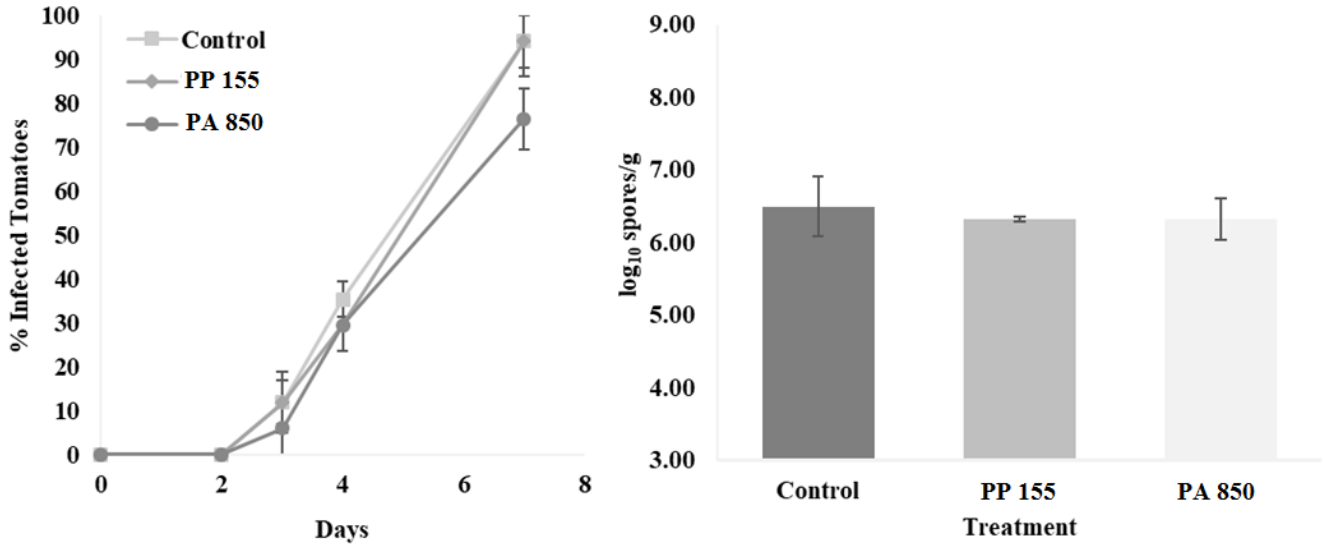
2,6-dimethyl pyrazine	9.56 ± 0.01	16.83 ± 0.08	7.57 ± 0.19	7.33 ± 0.01	9.37 ± 0.09	4.7 ± 0.03	5.22 ± 0.01	0.49 ± 0.03	3.36 ± 0.03	5.22 ± 0.01
2-ethyl- 6-methylpyrazine	1.57 ± 0.01	2.67 ± 0.01	2.89 ± 0.08	3.48 ± 0.03	1.07 ± 0.02	1.6 ± 0.03	1.21 ± 0.01	0.88 ± 0.01	0.53 ± 0.01	1.21 ± 0.01
2-ethyl- 5-methylpyrazine	1.37 ± 0.01	10.62 ± 0.02	2.24 ± 0.06	0.95 ± 0.01	1.98 ± 0.01	8.58 ± 0.06	10.64 ± 0.02	9.09 ± 0.03	10.42 ± 0.1	10.64 ± 0.02
trimethyl pyrazine	1.03 ± 0.01	nd	1.53 ± 0.04	1.34 ± 0.02	1.88 ± 0.01	nd	nd	nd	Nd	nd
2-ethenyl-6-methylpirazine	0.49 ± 0.01	0.52 ± 0.01	0.48 ± 0.01	0.4 ± 0.01	0.34 ± 0.01	nd	0.46 ± 0.01	0.11 ± 0.01	Nd	0.46 ± 0.01
3-ethyl-2,5-dimethylpyrazine	6.97 ± 0.01	1.66 ± 0.01	4.85 ± 0.14	21.17 ± 0.01	3.19 ± 0.01	3.52 ± 0.02	3.19 ± 0.02	2.89 ± 0.02	1.17 ± 0.01	3.19 ± 0.02
Tetramethylpyrazine	nd	11.84 ± 0.01	0.2 ± 0.01	0.26 ± 0.01	nd	7.87 ± 0.04	10.97 ± 0.08	12.8 ± 0.08	21.4 ± 0.13	10.97 ± 0.08
2 - ethyl-3,5-dimethylpyrazine	nd	nd	2.12 ± 2.43	0.13 ± 0.01	nd	1.94 ± 0.02	2.25 ± 0.03	4.07 ± 0.05	0.46 ± 0.01	2.25 ± 0.03
2,3-diethyl-5-methylpyrazine	0.24 ± 0.01	0.05 ± 0.01	0.13 ± 0.01	0.39 ± 0.01	0.1 ± 0.01	0.12 ± 0.01	0.14 ± 0.01	0.27 ± 0.01	0.03 ± 0.01	0.14 ± 0.01
3,5-diethyl-2-methylpyrazine	0.69 ± 0.01	0.16 ± 0.01	0.44 ± 0.01	2.48 ± 0.01	0.25 ± 0.01	7.08 ± 0.01	0.36 ± 0.01	18.41 ± 0.39	0.09 ± 0.01	0.36 ± 0.01
2,3,5-trimethyl-6-ethylpyrazine	0.26 ± 0.01	5.26 ± 0.02	0.27 ± 0.01	0.49 ± 0.01	0.2 ± 0.01	0.07 ± 0.01	6.13 ± 0.02	0.12 ± 0.01	13.6 ± 0.37	6.13 ± 0.02
2,5-dimethyl-3-isobutylpyrazine	0.62 ± 0.01	0.49 ± 0.01	0.74 ± 0.03	0.43 ± 0.01	0.58 ± 0.01	0.45 ± 0.01	0.65 ± 0.01	0.35 ± 0.01	0.25 ± 0.01	0.65 ± 0.01
2-acetyl-3,5-dimethylpyrazine	nd	nd	nd	N	nd	0.35 ± 0.01	0.53 ± 0.01	0.41 ± 0.01	0.43 ± 0.01	0.53 ± 0.01
2-isoamyl-6-methylpyrazine	0.71 ± 0.01	0.35 ± 0.01	0.35 ± 0.01	0.68 ± 0.01	0.32 ± 0.01	0.42 ± 0.01	0.53 ± 0.01	0.2 ± 0.01	0.08 ± 0.01	0.53 ± 0.01
3,5,6-trimethylpyrazine	nd	0.28 ± 0.01	nd	0 ± 0	nd	0.7 ± 0.01	0.7 ± 0.01	1.21 ± 0.01	Nd	0.7 ± 0.01
2,6-dimethyl-3 – isopenthylpyrazine	0.51 ± 0.01	0.16 ± 0.01	0.31 ± 0.01	0.15 ± 0.01	0.22 ± 0.01	0.22 ± 0.01	0.19 ± 0.01	0.13 ± 0.01	0.09 ± 0.01	0.19 ± 0.01
2,5-dimethyl-3-isopenthylpyrazine	18.96 ± 0.01	1.13 ± 0.01	1.36 ± 0.02	3 ± 0.02	0.75 ± 0.01	2.1 ± 0.02	2.73 ± 0.02	1.07 ± 0.01	0.38 ± 0.01	2.73 ± 0.02
2,3-dimethyl-5 iso-penthylpyrazine	nd	nd	nd	Nd	nd	0.91 ± 0.01	1.18 ± 0.01	0.8 ± 0.01	0.84 ± 0.01	1.18 ± 0.01
2,3,5-trimethyl-6-isopenthylpyrazine	0.02 ± 0.01	nd	0.12 ± 0.01	0.18 ± 0.01	nd	nd	nd	nd	Nd	nd
2,3,5-trimethyl-6-propylpyrazine	nd	3.49 ± 0.02	0.17 ± 0.01	Nd	nd	7.17 ± 0.04	8.35 ± 0.06	12.64 ± 0.06	16.33 ± 0.08	8.35 ± 0.06
OTHERS	23.45 ± 0.15^{f}	14.24 ± 0.02^{c}	21.91 ± 0.52^{e}	21.95 ± 0.05^{e}	27.48 ± 0.05^{g}	13.72 ± 0.59^{c}	15.97 ± 0.1^{d}	8.43 ± 0.04^{a}	10.27 ± 0.11^{b}	15.97 ± 0.1^{d}
2,4, di tert-butylphenol	16.82 ± 0.05	12.19 ± 0.01	18.37 ± 0.47	17.43 ± 0.04	22.91 ± 0.03	9.87 ± 0.52	9.74 ± 0.04	7.55 ± 0.03	7.96 ± 0.09	9.74 ± 0.04
2-t-butyl-phenol	4.79 ± 0.03	1.63 ± 0.01	1.81 ± 0.01	3.56 ± 0.01	1.8 ± 0.01	1.97 ± 0.05	4.54 ± 0.06	0.27 ± 0.01	0.51 ± 0.01	4.54 ± 0.06
Di tert-butyl-benzene	1.13 ± 0.01	0.43 ± 0.01	nd	0.83 ± 0.01	1.34 ± 0.01	1.51 ± 0.02	1.25 ± 0.01	0.44 ± 0.01	0.59 ± 0.01	1.25 ± 0.01
1,5-cyclooctadien, 1-6, dichloro	0.07 ± 0.08	nd	0.16 ± 0.01	0.15 ± 0.01	0.06 ± 0.01	nd	nd	nd	Nd	Nd
ethylacetate	0.68 ± 0.01	nd	1.58 ± 0.05	Nd	0.92 ± 0.01	nd	nd	nd	Nd	Nd
1-nonene	nd	nd	nd	Nd	0.47 ± 0.01	0.39 ± 0.01	0.46 ± 0.01	0.19 ± 0.01	1.22 ± 0.02	0.46 ± 0.01

Table 4c). Identification and quantification of Volatile Organic Compounds (VOC)(%) produced by LAB in CFS.

3.5 *Lactobacillus plantarum PP155* and *Lactobacillus plantarum PA850* as a tomato bio preservative

Since activity has been observed only for the *Lactobacillus plantarum PP155* and *Lactobacillus plantarum PA850* these two strains have been considered as case study using tomatoes as food matrix. Figure 3shows the bio-preservation effect of CFS fermented by *L. plantarum* PP155 and *L. plantarum* PA850on tomatoes inoculated with *A. flavus* and *P. expansum*, during storage. In particular, the visible shelf-life of tomato inoculated with *A. flavus* did not present a significant (p> 0.01) increase compared with the control. All treatment evidenced >70 % infected tomatoes after incubation for 7 d. Microbiological analysis of the population of the fungal confirmed the absence of inhibition of fungal growth in tomatoes treated with CFS. However, tomatoes inoculated with *P. expansum*and treated with CFS showed a visibly improved shelf-lifeas shown in Figure 4. In the control experiment, the percentage of infected tomatoes on day 9 of incubation was 100 %, whereas, when CFS fermented by *L. plantarum* PP155 and *L. plantarum* PA850 were used,the values were 29 % and 65 %, respectively. The observed shelf-life data of tomatoes inoculated with *P. expansum*and treated with CFS were confirmed by the microbiological analysis. The control experiment at 9 d of incubation, presented a fungal population of 7.56 $\log_{10}$ spores g^{-1}, whereas in the tomatoes treated with CFS fermented by *L. plantarum* PP155, significant (p< 0.01) fungal growth of 5.58 $\log_{10}$ spores g^{-1} was observed. In the treatment with CFS fermented by *L. plantarum* PP850, infected tomatoes incubated for 9 d, presented a significant (p< 0.01) fungal count of 3.67 $\log_{10}$ spores g^{-1}.

A)



B)

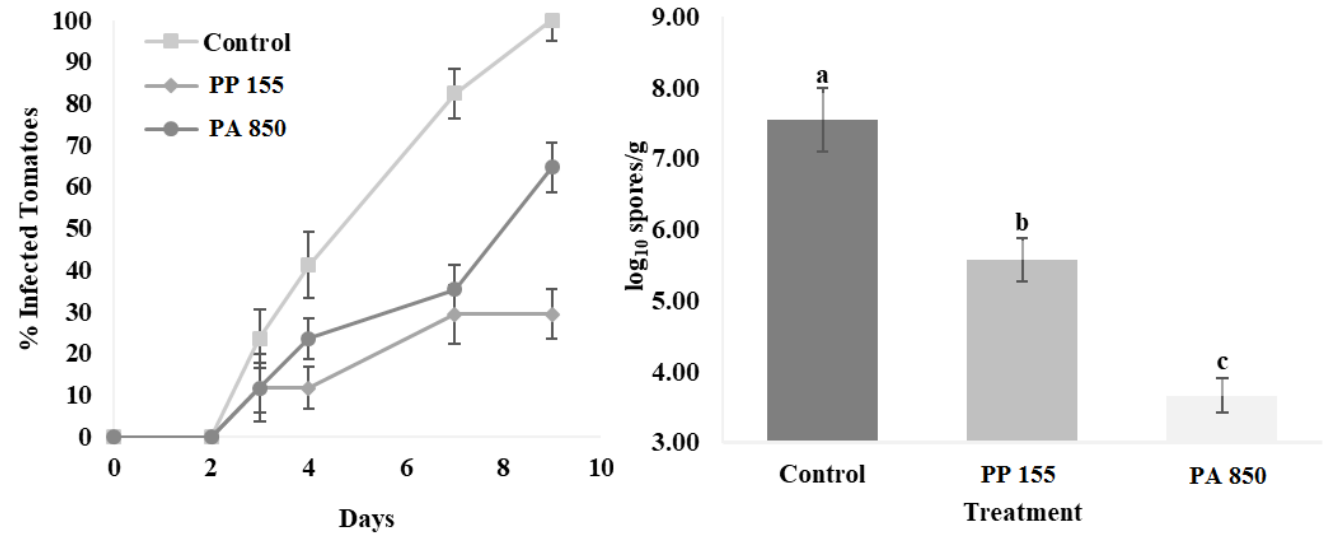


Figure 3. Effects of *Lactobacillus plantarum* PP155 and *Lactobacillus plantarum* PP850 CFS in growth of *Aspergillus flavus* ITEM 8111 A) and *Penicillium expansum* CECT 2278 on tomatoes B). Results are expressed as% of infected tomatoes and microbiological count (log_{10}spores g^{-1}). Statistically significant differences for each treatment are indicated with different letters ($p < 0.01$). The results are expressed as mean ± standard deviation.

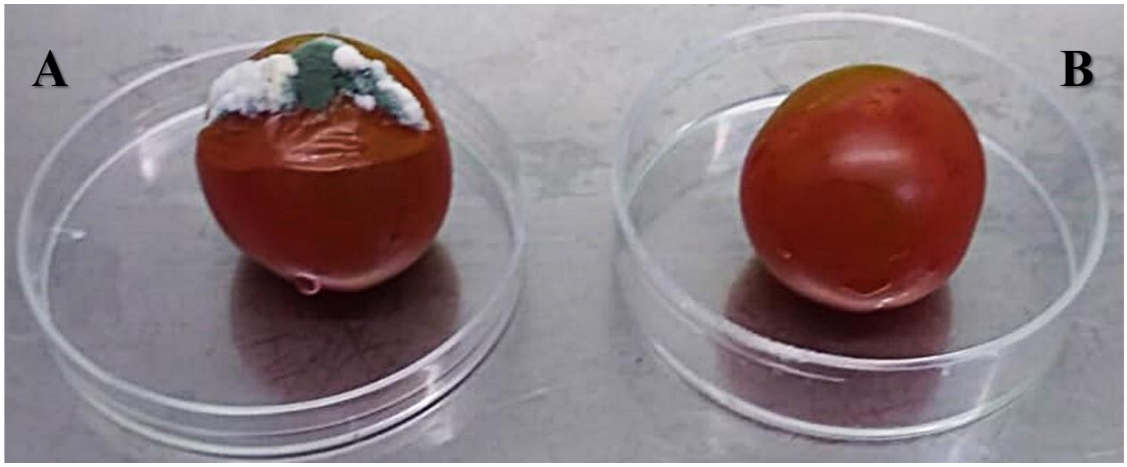


Figure 4. Fungal growth of *Penicillium expansum* CECT 2278 on tomato treated with sterile MRS Broth,**A**, and *Lactobacillus plantarum* PP850 CFS at 9 days of incubation, **B**.

Discussion

The possible mycotoxins contamination in raw materials and/or in transformed foods is a serious risk. The vegetal origin of phytocomplexes and possible mycotoxins occurrence may affect the processes of extraction and concentration of the active principles, representing a critical point to be monitored to provide, safe and mycotoxins free nutraceutical and/or functional food. Despite the efficiency of synthetic chemical compounds in eliminating mycotoxin-producing fungi and mycotoxin reduction, the residues of many chemicals pose health risks to humans and animals. Due to the toxicity of these exogenous xenobiotics used to reduce mycotoxin production and fungal growth, numerous studies have been conducted to identify effective natural alternatives.This part of the thesis provides insights into suitable alternativesto reduce also possible toxicological risk associatedwithmycotoxin-contaminated phytocomplexcomponents of a nutraceutical or functional food. From thispoint of viewthestudy utilized the properties of biocontrol agents. Antifungal *in vitro* experiments demonstrated that CFS fermented by different strains of *Lactobacillus* have significant antifungal activity against a broad spectrum of toxigenic fungi. These effects may be due to the bacteriocins and to the secondary metabolic compound produces by these strains. Furthermore, the application of CFS as a novel bio-preservative in tomatoes, chosen as a case study whose results can be extended to other vegetal food matrices, evidenced a reduction in the spoilage associated with *Penicilliumexpansum* growth.

Conclusion

The promising application presented in this thesis to decrease the microfungi contamination and increase the post-harvest shelf-life of tomatoes, is efficient to protect from the mycotoxins contamination. This contributes to meeting the demand of consumers to reduce the agricultural use of synthetic compounds and increase the choice of suitable natural alternatives.This study presents the importance of *Lactobacillus plantarum* spp. and the production during the fermentation of bioactive metabolites with antifungal activity. The strains assessed in this study could be considered as an strategy for inhibiting toxigenic fungi and as a substitute for synthetic compounds in food preservation and nutraceutical preparations.

Chapter III

Nutraceuticals formulations

First study: A Pilot Screening of Agro-Food Waste Products as Sources of Nutraceutical Formulations to Improve Simulated Postprandial Glycaemia and Insulinaemia in Healthy Subjects

1. Introduction

A wide range of natural substances of plant origin, specifically, polyphenols, carotenoids, and terpenoids have been demonstrated to be active on glycaemia in humans (Violi et al. 2017; Ylonen et al. 2003; Annunziata et al. 2019). To this regard, agro-food waste products are increasingly attracting a great interest from the nutraceutical industry, since they represent still rich sources of bioactive compounds which can be conveniently recovered for the formulation of food supplements and nutraceuticals indicated for the prevention and/or control of glycaemia. Figure1. summarizes, after the identification of a potential health condition target,the steps to follow for the development of a correct, safe and effective nutraceutical. Many different steps are involved, each requiring different professional expertise. The first step is the identification of the epidemiological target, followed by the identification and fractioning of the starting food matrix. The study of bioactivity and safety are a must, as well as identifying the metabolic profile of the phytocomplex. The study of the mechanism of actionis then followed by: (i) the establishment of the proper formulation; (ii) the fine tuning to develop the correct pharmaceutical form for the particular use and dosage; iii) the bioavailability determination of the nutraceutical being developed; iv) the assessment of appropriate clinical trials to substantiate its efficacy.

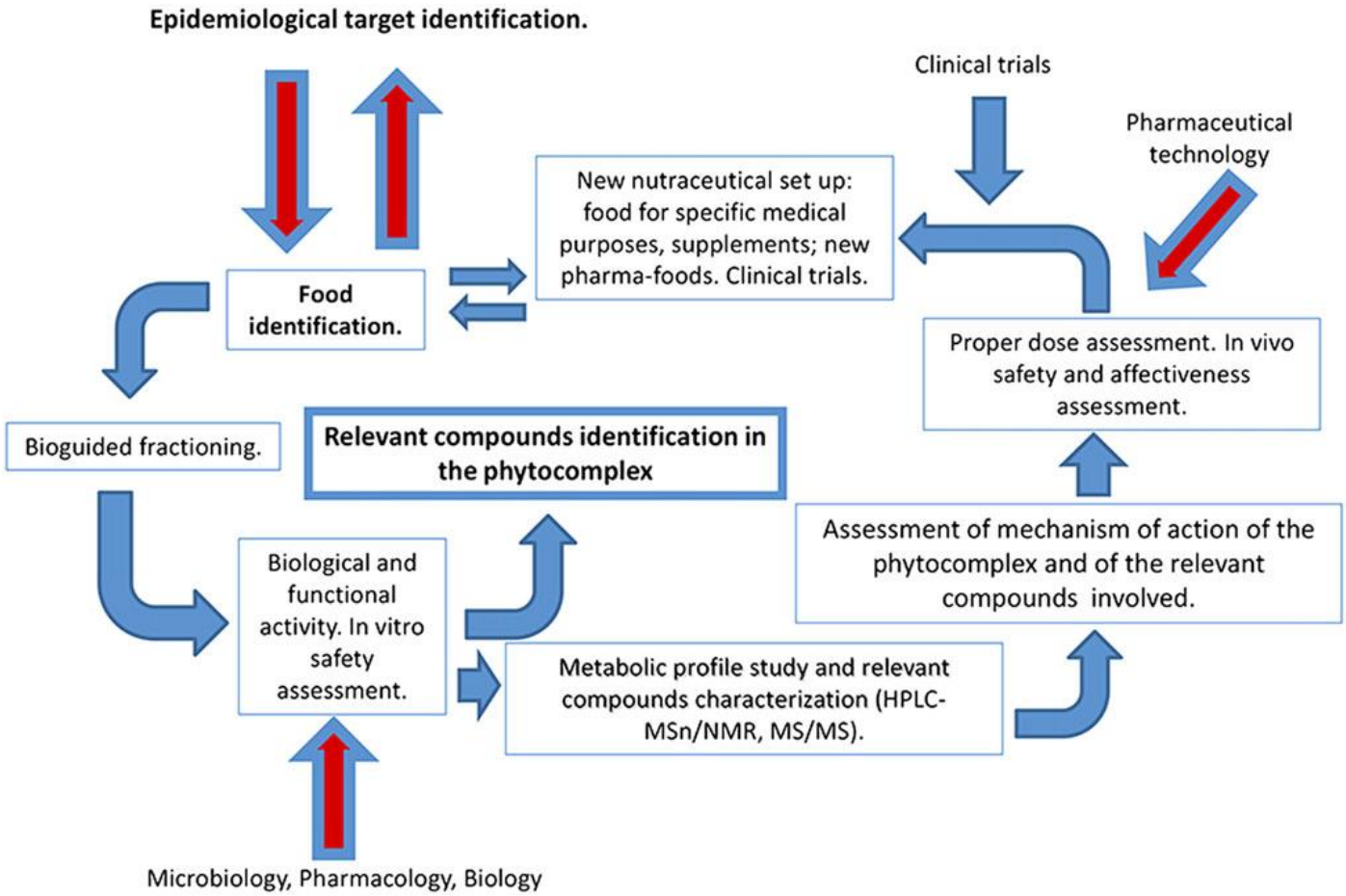


Figure 1. The steps to follow in a novel nutraceutical formulation and assessment.

The first aim of this first studyhas been to formulate pilot nutraceutical products based on: water extract of unripened fruits derived from fruit thinning (fruit thinning waste products, FTWPs); ethanol extract from olive leaves (EOL); dried tomato peel powder (TP). The extracts containedabscisic acid (ABA), oleuropein, and carotenoids, respectively. Each formulation has been tested on healthy human subjects in order to evaluate its effects on glycaemic and insulinemic responses compared to a standard glucose drink as detailed in the following.

Fruit thinning is a widespread agronomical practice that involves removing excess unripened fruits, since this practice may interest up to 40% of the entire tree fruit load. Fruit thinning may lead to a massive agricultural waste product (Lai et al. 2017). Interestingly, these waste fruits are a significant source of ABA. This phytohormone is majorly responsible for the regulation of plant growth and differentiation (Leng et al. 2014; Zhang et al. 2001; Ghosh et al. 2014). Studies have revealed that there is a progressive accumulation of ABA during fruit ripening, reaching its maximum concentration at a specific stage after full bloom and then decreasing to its minimum level at the fruit fully ripe/harvest stage (Magnone et al. 2015; Sun et al. 2012; Zhao et al. 2012). The plant hormone ABA is also produced by pancreatic β-cells adipocytes and myoblasts (Bruzzone et al. 2008), in response to glucose and active in humans as described previously in my thesis. The most recent studies concerning the mechanism of interaction of ABA with the receptor lanthionine synthetase C-like 2 (LANCL2) in glycaemic control and their influence on insulin and glucagon-

like peptide 1 (GLP-1) release, highlight a leading role of ABA in the physiological regulation of plasma glucose levels in humans (Zocchi et al. 2017). Therefore, the administration of ABA at low doses may be suggested as a useful tool for the improvement of glucose tolerance in diabetic patients with deficiency of or resistance to insulin. Encouraged by these data, we might consider the fruit thinning waste products as a convenient agro-food matrix for nutraceutical applications. Moreover, these formulations, being a source of ABA, would increase the intake of this human endogenous hormone, contributing to its plasma levels and, consequantly to its physiological capacity to modulate glycaemia.

Another matrixconsidered are the olive leaves, a massive agricultural by-product of the harvesting or processing technology of olive fruits. They contain high amounts of polyphenols beneficial to human health.Olive leaf extracts, have been used in traditional medicine since centuries for their properties. Oleuropein, one of the most abundant constituents of olive leaf extract,has been reported to improve postprandial glycaemia, by counteracting Nox2-mediated oxidative stress recognized as being among the main responsible for the cellular production of reactive oxygen species (ROS) (Violi et al. 2017).

Carnevale et al. 2018 demonstrated that 20 mg of pure oleuropein were able to lower postprandial glycaemia in healthy subjects by enhancing DPP-4 activity, plasma GLP-1, and insulin levels (Carnevale et al. 2018).

The third natural matrices involved in my research consisted of tomato by-product (tomato pomace), originates from industrial processing which represents up to 3% by fresh fruit weight, and consists mainly of peels, seeds and pulp. Nevertheless, the abundance of several bioactive compounds, especially carotenoids (mainly, lycopene), suggests the possibility of employing the tomato pomace as a cheap and sustainable source, for the extraction of these valuable natural substances.

Recent studies including clinical evidence of the bioactive properties of carotenoids have shown that these compounds may play a significant role in the treatment of diabetes by improving insulin resistance which has been indicated as a major risk factor for the development of type 2 diabetes mellitus (T2DM) (Sluijs et al 2015). Previous human clinical trials have indicated a linear correlation between plasma lycopene, β-carotene concentration and insulin sensitivity in healthy volunteers (Facchini et al. 2000; Sugiura et al. 2006). Specific carotenoids would act as peroxisome proliferator-activated receptor gamma (PPARγ) agonists, through a mechanismsimilartothiazolidinediones, a class of oral antidiabetic drugs, which are usedin clinical therapy (Takahashi et al. 2009). It has also been observed that carotenoid intake has an inverse relation with glycosylated hemoglobin (HbA1c) levels (Suzuki et al. 2002). Nevertheless, there is general agreement that the beneficial effects of carotenoids in diabetes cannot simply be associated with their antioxidant properties. Encouraged by these data, my aim has been to formulate a nutraceutical and start a clinical investigation to confirm the expected results by a clinical trial in healthy subject.

2. Materials and Methods

2.1. Standards and materials

All chemicals and reagents used were either analytical-reagent or high-performance liquid chromatography (HPLC) grade. The water was treated in a Milli-Q water purification system (Millipore, Bedford, MA) before use. (±)-2-Cis-4-trans-abscisic acid (ABA), cartridges Discovery SPE DSC-MCAX 6 mL; Supelco Analytical, Bellefonte PA, USA), Supelclean SPE LC-NH2 (bed wt, 300 mg; volume, 6 mL; Supelco Analytical), acetone, ammonium acetate, hexanes, methanol (MeOH), methyl tert-butyl ether (MtBE), β-Carotene (≥95%), and lycopene (≥90%), oleuropein (≥98%) were all purchased from Sigma–Aldrich (Milano, Italy). Glucose syrup 75 g 150 mL^{-1} was provided by Sclavo Diagnostics International S.r.l. (Sovicille, Siena, Italy).

2.2. Sample Collection and Preparation for HPLC Analyses

FTWPs (apples, pears, plums, peaches, and nectarines) were collected in June 2018 at the orchards of "GiaccioFrutta" Company (Vitulazio, Caserta, Italy), at 20-25 days after full bloom, at the fruit thinning stage. Samples were immersed in liquid nitrogen (N_2), and maintained at -80 °C until analysis. Then, samples were weighed and ground in liquid N_2, and 0.5 g of each homogenized sample was suspended in 15 mL of 80% aqueous methanol containing 1% formic acid for 16 h at 4 °C in darkness under magnetic stirring. The extract was diluted to 25 mL with water, and the pH was adjusted to 2.5 with 1 M aqueous HCl, after which the samples were filtered and eluted. The eluates were then added first to solid phase purification (SPE) using DSC-MCAX cartridges previously washed with 2 mL of 100% methanol and equilibrated with 2 mL of water. Next, the eluates (6 mL) were added to SPE LCNH2 type cartridges, also previously washed with 2 mL of 100% methanol, and equilibrated with 2 mL of water. The acidic compounds bound to the last column, were eluted with 1 mL of 5% phosphoric acid in methanol and stored at -20 °C until analysis.

Tomato peels (cultivar San Marzano) were provided in September 2018 by La Torrente s.r.l. Company (Angri, Salerno, Italy). Samples were immersed in liquid nitrogen (N_2), and maintained at -80 °C until analysis. Then, samples were weighed and ground in liquid N_2. Carotenoids were extracted in darkness, according to the procedure detailed in the following.

Approximately 1 g tomato peel powder was weighed into an 11 mL glass vial and extracted with 5mL MeOH, briefly mixed on a vortex mixer, probe sonicated (Branson Fisher Scientific 150E SonicDismembrator) for 8 s, and centrifuged for 5 min at 2000 rpm. The supernatant was decanted and the pellet was re-extracted with 5 mL hexane–acetone (1:1), briefly mixed on a vortex mixer, sonicated for 8 s, and centrifuged for 5 min at 2000 rpm. The supernatant was added to the methanolic extract, and the extraction was repeated two more times or until the pellet was colorless. To induce phase separation, 10 mL

water was added to the combined supernatants, 1 mL aliquots organic layer was filtered with a 0.22 μm nylon filter (CellTreat, Shirley, MA), and stored at -20 °C until analysis.

Olive leaves (cultivar Ravece) were provided in September 2018 by the AgriturismoPetrilli Company (Flumeri, Avellino, Italy). Samples were immersed in liquid nitrogen (N_2), and maintained at -80 °C until analysis. Then, samples were weighed and ground in liquid N_2.

Polyphenols were extracted in darkness, according to the procedure described byVioli et al.2017 with slight modifications. In particular, an aliquot (1.0 g) of olive leaves powder was extracted with 20 mL ethanol (70%), stirred well by piping for homogenization for one minute, then placed in the ultrasonic bath at (75 ± 2) °C, for 30 min, filtered with 0.45 μm filters and kept at -20 °C until use.

All the extractions mentioned above have beenrepeatedthree times.

2.3. HPLC-DAD Analyses of Samples

The chromatographic apparatus used consisted of a Jasco Extrema LC-4000 system (Jasco Inc., Easton, MD) provided with the following modular components: a vacuum degassing unit, a quaternary pump, an autoinjector, a column oven, a diode array detector photodiode array detector (DAD).

ABA determination, the chromatographic analyses were performed according to the procedure suggested by Bosco et al. 2103 with slight modifications. The column selected was a Kinetex® C18 column 100A (250 mm x 4.6 mm i.d., 5 μm) (Phenomenex, Torrance, CA, United States). The mobile phase was (A) acetonitrile and (B) aqueous phosphoric acid solution at pH 3.2. The column was equilibrated, and the column temperature was maintained at 40±0.1 °C. Separation was carried out by gradient elution with a constant flow rate of 0.5 mL/min. The gradient program was as follows: 5-70% A (0−75 min). After the gradient separation, the column was re-equilibrated with 70-100% A (75−86 min) for 5 min and 100-5% A in 20 min. An injection volume of 20 μL was used for each analysis. The standard solution of ABA was prepared in methanol and chromatographed to determine the retention time for each. ABA was monitored at 265 nm.

Carotenoids were analyzed as previously described by Cooperstone et al, 2015 with slight modifications. Separation was performed on a 4.6 × 250 mm, 3 μm particle size, C30 column (YMC Inc., Wilmington, NC, United States) maintained at 35 °C. A total of 10 μL of purified samples was injected. A gradient using solvent A (60% MeOH, 35% MtBE, 3% water, and 2% aqueous ammonium acetate (w/v)) and B (78% MtBE, 20% MeOH, and 2% aqueous ammonium acetate(w/v)), at a flow of 1.3 mL/min, was used as follows: 100% A to 64.4% A over 9 min, 64.4% A to 0% A over 5.5 min, a hold at 0% A for an additional 3.5 min, and a switch to 100% A for the remaining 3.5 min to recondition the column. Quantification was achieved using a six-point external calibration curve of lycopene and β-carotene. The other carotenoids have

been determined based on the ratios of their molar extinction coefficient compared tothe ones of lycopene, as reported by Cooperstone et al, 2015.

Oleuropein determination in olive leaves extracts was carried out as previously described AlShaal et al 2019, with minor modifications. A Kinetex® C18 column 100A (250 mm x 4.6 mm, i.d. 5 μm) (Phenomenex, Torrance, CA, United States) was used with the following gradient mobile phase program: A, 2.5% acetic acid; B, acetonitrile; flow rate, 1.0 mL/min. The temperature was set at 25 °C. The injection volume was 20 μL.The chromatograms were monitored at a wavelength of 254 nm, for the oleuropein determination.

For all of the three determination (ABA, carotenoids, oleuropein), the retention times were determined based on three different injections. Peak identifications has been based on the retention times with reference toappropriate standard. The compounds were quantified according to a calibration curve.

2.4. Nutraceutical formulations

Among all of the FTWPs objects of this study, nectarines were found to be the richest in ABA content as shown in Table 1. The formulation of nutraceuticals has been obtained as follows.

Nectarines (NecP)were extracted with water at 50 °C. After centrifugation, the extract underwent a spray-drying process with maltodextrins as support, obtaining a fine powder containing maltodextrins and NecP, in a 1:1 ratio (w/w).

Tomato peels were dried at 42 °C in industrial oven (OVEN STF-N 52 NATURAL VENTILATIONRef number 720.2080.05,IP 42, FALK ®) to obtain a fine dried tomato peel powder (TP).

Olive leaves were extracted with HPLC gradeethanolat room temperature. After centrifugation, the extract underwent a spray-drying process with maltodextrins as support, obtaining a fine powder, made ofethanol extract from olive leaves and maltodextrins (EOL)in a 1:1 ratio (w/w).

	Peaches	**Nectarines**	**Apples**	**Plums**	**Pears**
μg/g FW	0.9 ± 0.5^{a}	4.5 ± 1.5^{c}	0.8 ± 0.3^{a}	0.4 ± 0.2^{b}	0.3 ± 0.1^{b}
μg/g DW	9.5 ± 1.6^{a}	15.0 ± 3.0^{c}	8.1 ± 1.1^{a}	6.5 ± 0.9^{b}	5.5 ± 0.8^{b}

Table 1. Content of abscisic acid (ABA) in fruit thinning waste products.
Values are reported as the means ± SD ($n = 5$; $p < 0.01$). [abc]Mean values in rows with different superscript letters are significantly different by the Tukey–Kramer multiple comparison test. Abbreviations: FW, fresh weight; DW, dry weight

2.5. HPLC-DAD Analyses of Nutraceuticals

To determine the ABA, carotenoids, and oleuropein levels in the nutraceutical products,onNecP, TP, and EOL, chromatographic analyses were conducted as detailed in the Section 2.3. The results are shown in Table 2.

	Abscisic acid (from NecP)	Carotenoids (from TP)	Oleuropein (from EOL)
mg/g	0.007 ± 0.004	0.5 ± 0.1	150.0 ± 5.6

Table 2. HPLD-DAD determination of the main bioactive components in the nutraceutical products.

Values are the mean ± SD (n = 5; p< 0.01); Abbreviations: NecP, nectarine dry extract powder; TP, dried tomato peel powder; EOL, ethanol extract from olive leaves.

2.6 *Study treatment*

The reference glucose solutions and the treatment beverages all contained 75 g glucose. The three treatment beverages (TB) were prepared mixing glucose solutions with the following samples: 2 g of *NecP*(14 μg ABA) → TB *NecP*; 2 g TP (1.0 mg total carotenoids) → TB *TP*; 400 mg *EOL* (60.0 mg oleuropein) → TB *EOL*. Both TB and reference glucose solutions were served into dark jars, in order to blind subjects and researchers of the study to the different colours of the solutions mixed with the nutraceutical products. The nutraceutical products required for each treatment beverage was added into the glucose solution immediately before to being served to the subjects.

2.7. Study Population and Protocol

Arandomised, single centre, double-blind trial has been conducted on 18–70 years, normal-weight, and normal-glycaemic subjects recruited by the Samnium Medical Cooperative (Benevento, Italy) in January 2019.

Participants completed six test sessions, each on a different day with consecutive sessions, separated by at least 1 week. Each participant tested an oral glucose solution (contained 75 g glucose) on sessions 1, 3, and 5, and one of the three treatment beverages during each of the remaining sessions in a random, counterbalanced order (2 g of NecP (14 μg ABA) → TB NecP; 2 g TP (1.0 mg total carotenoids) → TB TP; 400 mg EOL (60.0 mg oleuropein) → TB EOL on one occasion only.Normal glucose tolerance was assessed using the results from an oral glucose tolerance test conducted within the previous 1 month prior to participation in this study (fasting glucose <5.5 mmol/L and 2 h

postprandial glucose <7.8 mmol/L).Subjects were informed not to drink alcohol or perform hard physical activity 48 h prior to blood sampling. Participants maintained their usual dietary and lifestyle patterns throughout the study. Both TB and reference glucose solutions were served into dark jars, in order to blind subjects and researchers of the study to the different colors of the solutions mixed with the nutraceutical tested. The study was conducted in accordance with the 1964 Helsinki Declaration (revised in 2000) and approved by the Scientific Ethics Committee of AO Rummo Hospital (Benevento, Italy) with protocol no. 28 of 15 May 2017.

2.8 Study procedures

Participants arrived at the research centre in the morning after 12 h of fasting. All blood samples were taken in the morning and immediately after measurement of heart rate and blood pressure.
Blood samples were collected from each participant before administration of the reference glucose solutions and the treatment beverages, in 3-mL EDTA-coated tubes (Becton–Dickinson, Plymouth, UK). Plasma was immediately isolated by centrifugation (20 min, 2.200 g, 4 °C). Additional fingerprick blood samples were collected at 30, 60, 90, 120 and 150 min after starting consumption of the reference glucose solutions and the TB. All seven test sessions for each participant were analysed within the same assay. All samples were stored at -80 °C until analysis. Plasma glucose levels were determined using commercially available kits from Diacron International (Grosseto, Italy). Analyses were performed on a Diacron International Free Carpe Diem. The assay sensitivity for the individual analytical determination was determined as follows: glucose, 4 mg/dL. Intra- and inter-day variations were 1.1 and 1.7% for glucose. Plasma insulin concentrations were measured using an enzyme linked immunosorbent (ELISA) assay commercial kit (InterMedicalsrl, Italy). The reference glucose solutions and the treatment beverages were consumed within 5 min.

2.9 Statistics

All of the experimental data were expressed as mean ± standard deviation (SD) of at least five replications. Statistical analysis of data was carried out by the Student's t test or two-way ANOVA followed by the Tukey-Kramer multiple comparison test to evaluate significant differences between a pair of means. The level of significance (α-value) was 95% in all cases ($P < 0.05$). The degree of linear relationship between two variables was measured using the Pearson product moment correlation coefficient (R). Correlation coefficients (R) were calculated using Microsoft Office Excel.

3. Results

3.1. Enrollment

A total of 20 healthy subjects (11 women and 9 men) with a mean age of 45.1 ± 15.8 years and an average BMI of 23.3 ± 3.4 kg/m^2 were assigned to the study. Table 3 reports the baseline values for the partecipants to the study. The group was well balanced for demographics and clinical factors. No subject prematurely terminated study participation. All participants performed the six test sessions (dropout rate: 0%). The mean within-individual coefficient of variation for the glycaemic responses to the three repeated glucose solutions was 11% which was within the accepted level of ≤30% (ISO 26642:2010).

Characteristics	**Value**
Demographics	
Age (years)	45.1 ± 15.8
Male sex (%)	9 (45.0%)
Female sex (%)	11 (55%)
White ethnicity (%)	20 (100%)
Clinical parameters	
BMI (Kg/m^2)	23.3 ± 3.4
TC (mg/dL)	190.1 ± 11.2
LDL-C (mg/dL)	98.0 ± 10.1
HDL-C (mg/dL)	57.2 ± 8.5
Triglycerides (mg/dL)	147.3 ± 12.7
Glucose (mg/dL)	82.5 ± 14.2

Table 3. Baseline characteristics of randomised subjects.

3.2. Tolerability of Treatment Beverages

The TB were palatable and well tolerated. No adverse events were reported.

3.3. Glycaemia and Insulinaemia Responses to Reference Glucose Solution and Treatment Beverages

All of the three TB revealed lower peak plasma glucose concentrations at 30 min compared to the reference glucose solution (TB NecP, $p = 0.02$; TB TP, $p = 0.02$; TB EOL, $p = 0.02$) (Figure 1). Particularly, TB TP, and TB EOL demonstrated higher effects respect to TB NecP ($p = 0.02$ and $p = 0.02$, respectively), showing no significant difference among each other ($p = 0.48$).

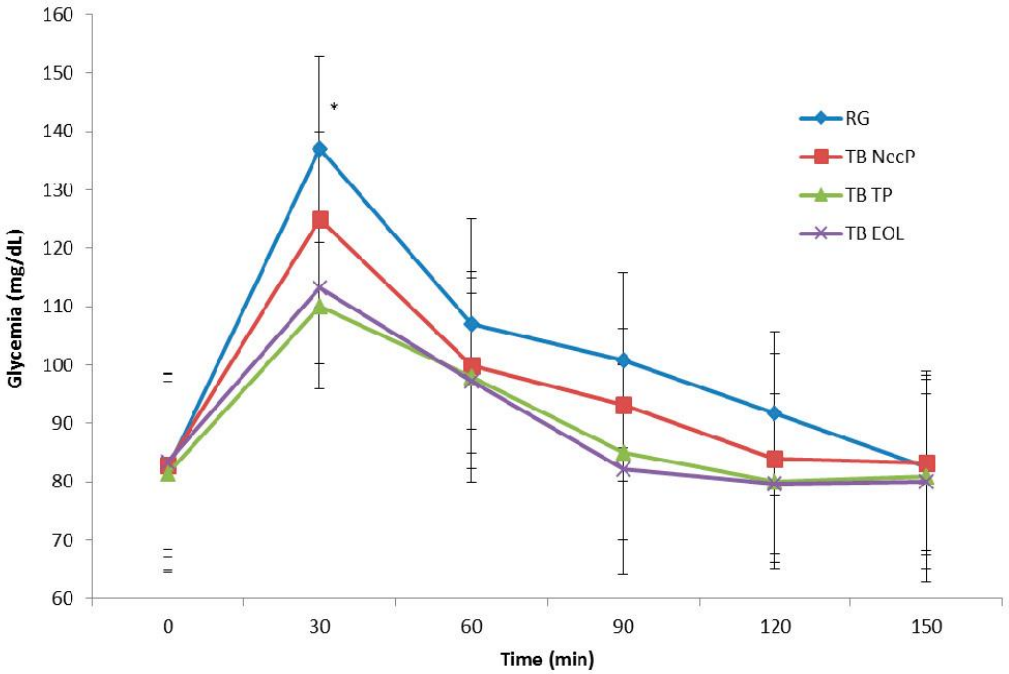


Figure 1. Change in postprandial plasma glucose concentration in healthy adults for the three treatment beverages. TB NecP (2 g of NecP, equivalent to 14 µg ABA); TB EOL (400 mg EOL, equivalent to 60.0 mg oleuropein); TB TP (2 g TP, equivalent to 1.0 mg total carotenoids). Data are mean ± SD. * Indicates a significant difference between peak 30 min glucose concentration for each treatment test compared to the reference test ($p < 0.05$). Abbreviations: RG, regular glucose solution; NecP, nectarine dry extract powder; TP, dried tomato peel powder; EOL, ethanol extract from olive leaves.

Reagarding the postprandial insulin response curves (Figure 2), TB NecP and TB TP produced lower peak plasma concentrations at 30 min respect to the reference glucose solution ($p = 0.03$ and $P = 0.04$, respectively), whereas TB NecP demonstrated the lowest effect compared to TB TP ($p = 0.03$). Conversely, TB EOL led to a higher peak insulin glucose concentration compared to the reference glucose solution ($p = 0.02$).

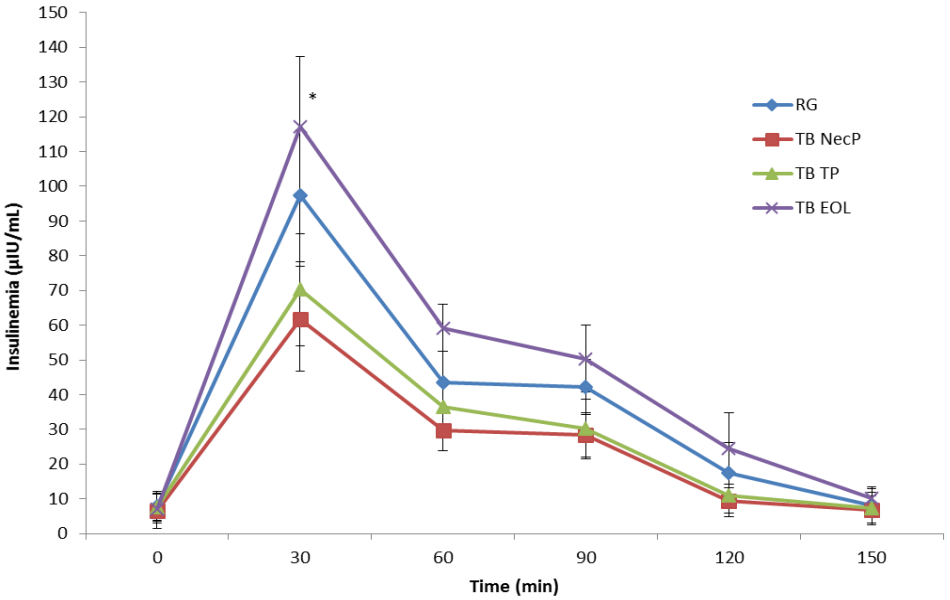


Figure 2. Change in postprandial plasma insulin concentration in healthy adults for the three treatment beverages. TB NecP (2 g of NecP, equivalent to 14 µg ABA); TB EOL (400 mg EOL, equivalent to 60.0 mg oleuropein); TB TP (2 g TP, equivalent to 1.0 mg total carotenoids). Data are mean ± SD. * Indicates a significant difference between peak 30 min insulin concentration for each treatment test compared to the reference test ($p < 0.05$). Abbreviations: RG, regular glucose solution; NecP, nectarine dry extract powder; TP, dried tomato peel powder; EOL, ethanol extract from olive leaves.

The total glucose response over 150 min was expressed as the postprandial glucose incremental area under the curve (iAUC) ignoring the area under the baseline using the trapezoidal rule by Brouns et al. 2015. All of the three TB produced lower postprandial glucose iAUC compared to the reference glucose solution (TB NecP versus RG, 8317 mg $dL^{-1}min^{-1}$ versus 9378 mg $dL^{-1}min^{-1}$, p=0.02; TB TP versus RG, 7558 mg $dL^{-1}min^{-1}$vs. 9378 mg $dL^{-1}min^{-1}$, p = 0.02; TB EOL vs. RG, 7611 mg $dL^{-1}min^{-1}$vs. 9378 mg $dL^{-1}min^{-1}$, p=0.02) (Figure 3). In particular, TB TP and TB EOL showed a higher effect with respect to TB NecP (p=0.02 and p = 0.02, respectively).TB TP and TB EOL showed no significant difference between each other (p=0.48).

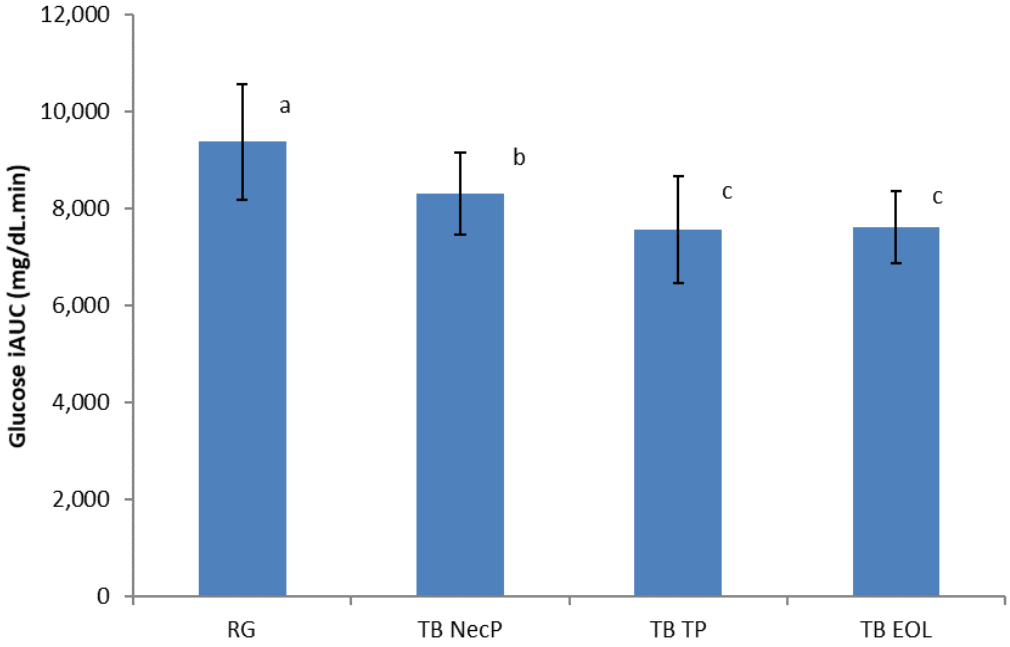


Figure 3. Incremental area under the curve (iAUC) postprandial glucose responses for the threetreatment beverages. TB NecP (2 g of NecP, equivalent to 14 µg ABA), TB EOL (400 mg EOL,equivalent to 60.0 mg oleuropein), and TB TP (2 g TP, equivalent to 1.0 mg total carotenoids)compared to the reference glucose solution. Data are mean ± SD. abc Mean values with differentsuperscript letters are significantly different by the Tukey–Kramer multiple comparison test (p <0.05). Abbreviations: RG, regular glucose solution; NecP, nectarine dry extract powder; TP, driedtomato peel powder; EOL, ethanol extract from olive leaves.

The postprandial insulin iAUC was calculated in the same manner as for the postprandial glucose iAUC, using the trapezoidal rule described by Tan el. 2015. As shown in Figure 4, TB NecP and TB TP produced lower effects respect to the reference glucose solution (TB NecP versus RG, 3572 µIUmL^{-1}min^{-1}versus 5649 µIU mL^{-1}min^{-1}, p = 0.03; TB TP versus RG, 4116 µIUmL^{-1}min^{-1}versus 5649 µIUmL^{-1}min^{-1}, p = 0.02), whereas TB NecP demonstrated the lowest effect compared to TB TP (p = 0.03). Conversely, TB EOL led to a higher postprandial insulin iAUC compared to the reference glucose solution (p = 0.02).

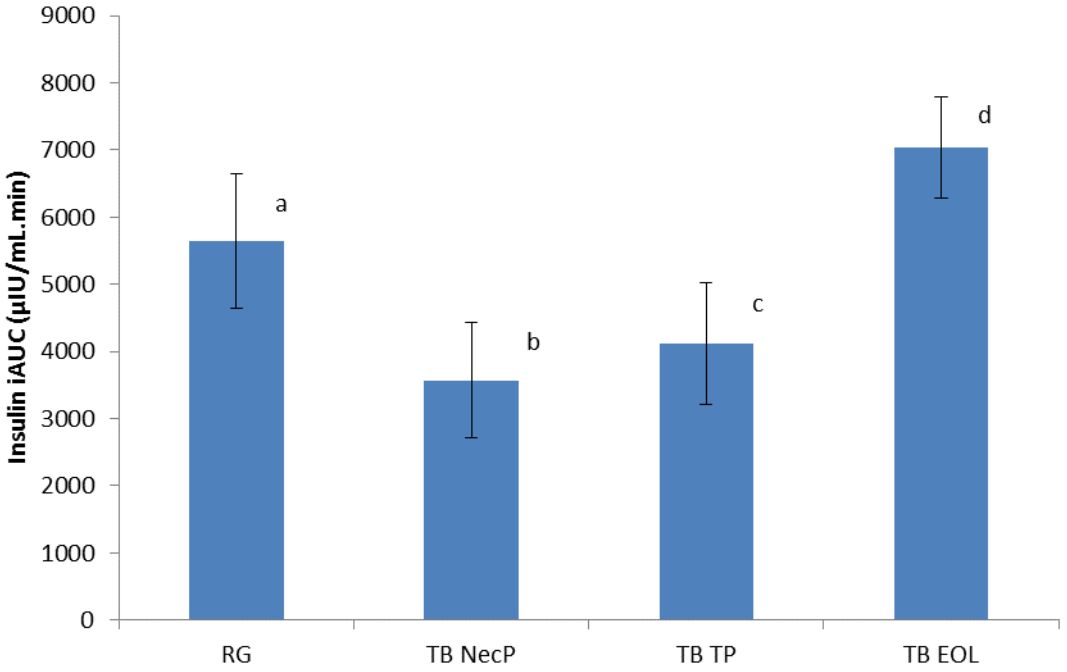


Figure 4. Incremental area under the curve (iAUC) postprandial insulin responses for the three treatment beverages.TBNecP (2 g of NecP, equivalent to 14 µg ABA), TB EOL (400 mg EOL, equivalent to 60.0 mg oleuropein), and TB TP (2 g TP, equivalent to 1.0 mg total carotenoids) compared to the reference glucose solution. Data are mean ± SD. [abcd] Mean values with different superscript letters are significantly different by the Tukey–Kramer multiple comparison test ($p < 0.05$). Abbreviations:RG, regular glucose solution; NecP, nectarine dry extract powder; TP, dried tomato peel powder; EOL, ethanol extract from olive leaves.

3.4. Glycaemic Index and Insulinemic Index of Treatment Beverages

For the calculation of the glycaemic index (GI), the absolute iAUC glucose value for each TB was expressed as a percentage of the mean iAUC glucose values of the standard glucose solution, and the resulting values were averaged to obtain the GI value for each TB (Tan et al. 2015). All of the three TBs led to significant reductions in GI compared to the reference glucose solution (TB NecP, $p = 0.02$; TB TP, $p = 0.02$; TB EOL, $p = 0.02$) (Table 4). Specifically, TB TP and TB EOL demonstrated higher effects respect to TB NecP ($p = 0.02$ and $p = 0.02$, respectively), showing no significant difference among each other ($p = 0.48$).

The insulinemic index (II) was calculated in the same manner as the GI, using the absolute iAUC insulin values as described by Tan et al. 2015. Table 4 reports the values of TB NecP and TB TP showing lower values with respect to the reference glucose solution ($p = 0.03$ and $p = 0.04$, respectively), whereas TB NecP demonstrated the lowest effect compared to TB TP ($p = 0.03$). Conversely, TB EOL led to a higher II compared to the reference glucose solution ($p = 0.02$).

Test solution	GI value	II value
RG	100[a]	100[a]
NecP	90 ± 5[b]	63 ± 4[b]
EOL	80 ± 4[c]	120 ± 7[c]
TP	83 ± 5[c]	72 ± 6[d]

Table 4. Glycaemic index (GI) and insulinemic index (II) for the three treatment beverages. TB NecP (2 g of NecP, equivalent to 14 µg ABA), TB EOL (400 mg EOL, equivalent to 60.0 mg oleuropein), TB TP (2 g TP, equivalent to 1.0 mg total carotenoids) in relation to the reference glucose solution (RG). Data are mean ± SD. [abcd] Mean values in columns with different superscript letters are significantly different by the Tukey–Kramer multiple comparison test ($p < 0.05$). Abbreviations: NecP, nectarine dry extract powder; TP, dried tomato peel powder; EOL, ethanol extract from olive leaves.

3.5. Insulin Sensitivity of Subjects in Response to Reference Glucose Solution and Treatment Beverages

Insulin sensitivity of subjects was evaluated in reference to data of glucose tolerance (Figure 1) and insulin secretion (Figure 2), and expressed as values of Matsuda Indexes (Matsuda and DeFronzo, 1999)as follows: RG, 5.99; TB NecP, 8.31; TB TP, 7.61; TB EOL, 5.98. The samples TB NecP and TB TP improved insulin sensitivity compared to the reference glucose solution in the treatment of subjects.

3.6. Study Strength and Limitations

The major strengths of the clinical trial herein presented reside in its originality and in the evaluation of the treatment effects. The positive results obtained can be useful to physicians about a novel treatment and or intervention whichmay represent a valuable support and or alternative in the clinical practice. The main limitations of our study which should be considered are: i) the small sample size of healthy participants with normal glucose tolerance and insulin sensitivity; ii) the short-term assessment for the treatment of a chronic condition which only allowed the investigation of acute postprandial effects of the nutraceutical formulations; iii) the lack of a dose assessment in order to define the range of minimum effective–maximum non-toxic concentrations of therapeutic interest.

4. Discussion

The obtained results revealed that all of the three nutraceutical formulations were able to significantly lower simulated postprandial glycaemia in healthy adults when added to the reference glucose solution (average reduction of glucose peak at 30 min: NecP, −9%; EOL, −17%; TP, −20%) (Figure 1).

Regarding the prediabetes management, previous authors reported that the occurrence of T2DM may be significantly decreased with a minimum lowering of 10 GI units through diet and/or the use of food supplements (Livesey et al. 2019). The study presented here indicates that the acute consumption of NecP, TP, and EOL may lead to an average decrease of 10, 17, and 20 GI units, respectively (Table 3).

Our results indicated lowering effects due to the treatmentbeverages on simulated postprandial insulinaemia in comparison with the reference glucose solution with the exception of TB EOL (average variation of insulin peak at 30 min: NecP, −28%; TP, −36%; EOL, +20%; average variation of the II: NecP, −37 units; TP, −28 units; EOL, +20 units) (Figure 2 and Table 3). Thus, the present study of my thesis would highlight that two out of three of the nutraceutical formulations (namelyNecP and TP) would be able to influence postprandial glycaemia through an insulin-saving mechanism, while EOL would preferentially modulate plasma glucose levels by stimulating insulin release.

It must be remarked that these nutraceutical formulations are characterized by very heterogeneous phytocomplexes. Our results regarding their influence on human glycemia and insulinemia could be ascribed to a plethora of different phytochemicals rather than to an individual constituent or a specific class of compounds. Considering these data and the many different mechanisms of action of NecP, TP, and EOL, we may hypothesize the formulation of a multi-component synergic product, potentially useful to modulate glycaemia and insulinaemia in multifactorial patients such as diabetic subjects.

5. Conclusions

This study is in line with the current worldwide trend to recover such agro-food wastes for environmental, economic, and healthy purposes. The results indicate that all of the nutraceutical formulations obtained from agro-foodby-products were clinically able to significantly reduce simulated postprandial glucose levels. With reference to the postprandial insulin responses, NecP and TP produced a lower peak plasma concentration, while EOL led to a higher peak insulin concentration, compared to the reference glucose solution. Two of the three nutraceutical formulations were able to influence postprandial glycaemia through an insulin-saving mechanism, while EOL would preferentially modulate plasma glucose levels by stimulating the insulin release. The difference in the mechanisms of action, due to the different main bioactive constituents of the three nutraceutical formulations, may lead to the hypothesis that a multi-component synergistic preparation could be considered an innovative and promising tool the management of postprandial glucose homeostasis in pre-diabetic and, possibly, diabetic subjects.

Second study: An evaluation of a nutraceutical contained Abscisic acid in patients with prediabetes

1. Introduction

The proposedstudy, has been encouraged by the results obtained until now from previous described research, and aimed to evaluate in particularthe effect of abscisic acid contained in nectarinpeach.This substance has been identifiedin my research thesis work for the first time in this vegetal matrix. The following study is addressed to the regression of impaired fasting glucose (IFG) or impaired glucose tolerance (IGT) conditions. To assess this aspect a clinical randomized trial based on sixtyfive patients with IFG or IGT has been conducted and ABA or placebo has been administeredfor 3 months. Gluco-metabolic parameters were screening during the study. Prediabetes is a disease conditionpreceeding the onset of diabetes and includes impaired fasting glucose (IFG) or impaired glucose tolerance (IGT) conditions. The main pathophysiology features of IFG and IGT are insulin resistance and reduced functionality of the pancreatic β-cells (Kanat et al. 2015). It has been observed that almost all type 2 diabetic patients go across prediabetes phase whose average duration is about 10 years (Bertram and Vos 2010). During this long transition step it is possible to avoid the evolution towards diabetes if proper interventions are adopted. Triggered by these evidence, the develop of a sustainable and novel nutraceutical approach, where the treatment goal should be the return to euglycemic condition, represent a challenge exploited in the following of my thesis. Ithas been shown that restoration and maintenance of normal glycemia values during prediabetes and early stages of type 2 diabetes (T2DM) can determine a long-term remission (Glechner et al. 2018). It has also been reported that some nutraceuticals are effective and safe in improving insulin sensitivity and glycemic control in patients with dysglycemia (Derosa2019a; Derosa 2020b). As reported in previous parts of this thesis, ABA,a triterpenoid phytohormone, has attracted a considerable interest due to its involvement in management of glucose homeostasis in humans (Zocchi et al.2017). Moreover, at nanomolar concentrations, ABA regulates insulin secretion intensifying its glucose-dependent release and stimulating glucose-independent one (Bruzzone et al. 2008). It has been reported that hyperglycemia produced an increase of ABA plasma levels in healthy subjects subjected to an oral glucose tolerance test (OGTT), and that this phytormone, at nanomolar concentrations, promoted peripheral glucose uptake in adipocytes and myoblasts, similarly to insulin (Bruzzone et al. 2012). In the next sections will described in greater detail the evidences accumulated until now from our screening, which expands the view and highlights our attention for the role ABA in the prevention /treatment of type II diabetes as a regulator of glucose metabolism and encouragement for further investigations.

2. Materials and methods

2.1. Sample preparation and nutraceutical formulation

Nectarines thinning waste were collected in June 2018 at the orchards of "GiaccioFrutta" Company (Vitulazio, Caserta, Italy), at 20-25 days after full bloom, at the fruit thinning stage. Samples were immersed in liquid nitrogen (N_2), and maintained at -80 °C until analysis. Then, were weighed and ground in liquid N_2, and 0.5 g of each homogenized sample was suspended in 15 mL of 80% aqueous methanol containing 1% formic acid for 16 h at 4 °C in darkness under magnetic stirring. The extract was diluted to 25 mL with water, and the pH was adjusted to 2.5 with 1 M aqueous HCl, after which the samples were filtered and eluted. The formulation of nutraceuticals has been obtained as follows.

To determine the presenc of ABA in nectarin thinning waste, chromatographic analyses by HPLC-DAD was conducted as detailed in the previously in the first study of this thesis, according to the procedure suggested by Bosco et al. 2103 with slight modifications.Nectarines (NecP) were extracted with water at 50 °C. After centrifugation, the extract underwent a spray-drying process with maltodextrins as support, obtaining a fine powder containing maltodextrins and NecP, in a 1:1 ratio (w/w) named "ABA nutraceutical". This has been formulated in plastic sachet before the administration per os.

2.2. Study design and patients

A three-months, double-blind, randomized, placebo-controlled, clinical trial has been conducted at the Department of Internal Medicine and Therapeutics, University of Pavia and at the Department of Pharmacy, University of Napoli Federico II, Italy. The study protocol was approved by Institutional Ethical Committee (P-2017000837) and was conducted in accordance with the 1994 Declaration of Helsinki [16], and its amendments and the Code of Good Clinical Practice. All patients provided written informed consent to participate in this study after a full explanation of the study.

We enrolled patients with IFG or IGT, not taking hypoglycemic agents (both pharmaceutical or nutraceutical agents). Suitable patients, identified from review of case notes and/or computerized clinic registers, were contacted by the investigators in person or by telephone.

Patients were excluded if they had type 1 or type 2 diabetes mellitus, impaired hepatic function (defined as plasma aminotransferase and/or gamma-glutamil transpeptidase (γ-GT) level higher than the three times the upper limit of normal [ULN] for age and sex), impaired renal function (defined as serum creatinine level higher than the ULN for age and sex), or gastrointestinal disorders; current or previous evidence of ischemic heart disease, heart failure, or stroke; weight change of >3 Kg during the preceding 3 months; malignancy, and significant neurological or psychiatric disturbances, including alcohol or drug abuse. Excluded medications (within the previous 3 months) included hypoglycemic agents, laxatives, β-agonists (other than inhalers), cyproheptadine, anti-depressants, anti-serotoninergics, phenothiazines, barbiturates, oral

corticosteroids, and anti-psychotics. Women who were pregnant or breastfeeding or of childbearing potential and not taking adequate contraceptive precautions were also excluded.

2.3. Treatments

Patients were randomized to placebo or ABA nutraceutical formulation (2 g of lyophilized extract from dwarf nectarines, corresponding with 14 μg of abscisic acid) for 3 months. Both ABA and placebo were self-administered three times a day, 1 sachet before the breakfast, 1 sachet before the lunch, and 2 sachets before the dinner.

Both ABA nutraceuticals and placebo were supplied as identical sachets with coded to ensure the blind status of the study. Randomisation was done using a drawing of envelopes each given a code. Medication compliance was assessed by counting the number of empty sachets returned at the time of specified clinic visits. All ABA containing nutraceuticals were provided free of charge.

2.4. Assessments

At the study beginning, all patients underwent an initial screening assessment including a medical history, physical examination, vital signs (blood pressure and heart rate), a 12-lead electrocardiogram, measurements of height and body weight, calculation of body mass index (BMI), evaluation of fasting plasma glucose (FPG), post-prandial plasma glucose (PPG), glycated hemoglobin (HbA_{1c}), fasting plasma insulin (FPI), homeostatic model assessment of insulin resistance (HOMA index), total cholesterol (TC), low density lipoprotein-cholesterol (LDL-C), high density lipoprotein-cholesterol (HDL-C), triglycerides (Tg), aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transpeptidase (γ-GT), creatinine, and high sensitivity C-reactive protein (Hs-CRP).

All parameters were evaluated at baseline and after 3 months since the study start. Moreover, at baseline, and after 3 months, patients underwent an oral glucose tolerance test (OGTT), an euglycemic hyperinsulinemic clamp, and a glucagon test.

2.5. Glucagon stimulation test technique

Glucagon test were performed before randomization, and at the end of the study. Between 8:00 am and 8:30 am, Glucagon (Novo Nordisk A/S, Bagsværd, DENMARK) was injected into the antecubital vein (1 mg) within 2 minutes after an overnight fast. Patients did not receive any ABA containing nutraceutical in the morning of the study. Blood samples were taken from the other arm at baseline (time 0) and after 6 minutes from the glucagon injection (Miki et al. 1992).

2.6. Statistical analysis

An intention-to-treat (ITT) analysis has been conducted in patients who received ≥1 dose of the nutraceutical containing ABA and had a subsequent efficacy observation. Patients were included in the tolerability analysis if they had received ≥1 dose of trial medication after randomization and had undergone a subsequent tolerability observation. A two-way repeated measures analysis of variance (ANOVA) to test continuous variables has been used. Intervention effects were adjusted for additional potential confounders using analysis of covariance. An analysis of variance to assess the significance within and between groups has been conducted. The null hypothesis that the expected mean glycemia change from the end of the study did not differ significantly between placebo, and the nutraceutical was tested using a two-way repeated measures analysis of variance (ANOVA) model.Similar analyses were applied to the other variables. A 1-sample t-test was used to compare values obtained before and after treatment administration; 2-sample t-tests were used for between-group comparisons (Winer 1971). Statistical analysis of data was performed using the Statistical Package for Social Sciences software version 14.0 (SPSS Inc., Chicago, Illinois, USA). Data are presented as mean (SD). For all statistical analyses, $p< 0.05$ was considered statistically significant.

Results

3.1. Study sample

On the total of sixtyfive patients enrolled in the trial, thirtythreewere randomized to take ABA containing nutraceutical, and thirtytwo to take the placebo. Sixty patients completed the study; there were 5 patients who did not complete the study and the reasons for premature withdrawal included non-compliance to treatment (1 male in ABA group, and 1 female in placebo group, respectively) or lost to follow-up (1 male in placebo group and 2 females in ABA group, respectively) as shown in Figure 1. The characteristics of the patient population at the time of the start and during the study are shown in Table 1 and Table 2.

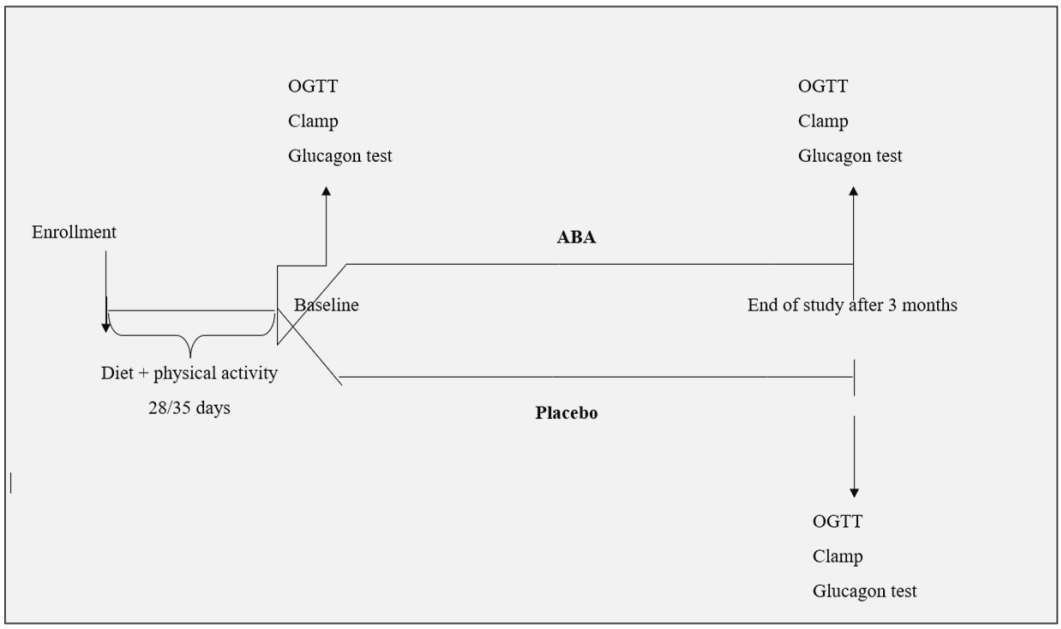


Figure 1. Study design. **OGTT:** oral glucose tolerance test; **Clamp:** euglycemic hyperinsulinemic clamp.

Parameters	**ABA**		**Placebo**	
	Baseline	**3 months**	**Baseline**	**3 months**
Patients (n)	33	30	32	30
M/F	15/18	14/16	16/16	15/15
Age (years)	51.9 ± 6.5	-	52.2 ± 6.8	-
Smoking status (M/F)	7/6	6/5	8/6	6/6
IFG (n; %)	6/7 (39.4)	3/3 (26.7)	5/6 (34.4)	4/5 (30.0)
IGT (n; %)	9/11 (60.6)	3/10 (46.7)	11/10 (65.6)	10/9 (63.3)
EU from IFG (n; %)	-	5/3 (61.5)	-	0/0
EU from IGT (n; %)	-	0	-	0/0

IFG from IGT (n; %)	-	0/0	-	1/0 (4.8)
IGT from IFG (n: %)	-	3/0 (23.1)	-	0/0
D from IFG (n; %)	-	0/0	-	0/0
D from IGT (n; %)	-	0/0	-	1/1 (9.5)
Lost to FU from IFG (n; %)	-	1/1 (15.4)	-	0/1 (9.1)
Lost to FU from IGT (n; %)	-	0/1 (7.7)	-	1/0 (4.7)

Table 1. Baseline, and 3-month general data of patients during ABA nutraceutical (ABA) treatment and placebo. M: males; F: females; IFG: impaired fasting glycemia; IGT: impaired glucose tolerance; EU: euglycemia; D: diabetes; FU: follow-up.

Parameters	**ABA**		**Placebo**	
	Baseline	**3 months**	**Baseline**	**3 months**
Height (cm)	169 ± 0.05	-	168 ± 0.04	-
Weight (Kg)	77.4 ± 6.1	76.5 ± 5.9	77.1 ± 5.9	76.2 ± 5.7
BMI (Kg/m^2)	27.1 ± 1.3	26.8 ± 1.1	27.3 ± 1.5	27.0 ± 1.2
WC (cm)	86.5 ± 4.9	86.4 ± 4.8	86.8 ± 5.0	86.7 ± 4.9
HC (cm)	89.2 ± 5.2	89.0 ± 5.0	88.9 ± 4.9	88.7 ± 4.7
AC (cm)	97.2 ± 5.8	97.0 ± 5.6	97.4 ± 6.0	97.2 ± 5.8
FPG (mg/dl)	109.4 ± 6.5	104.5 ± 6.1*^	112.8 ± 5.6	110.7 ± 5.5
PPG (mg/dl)	144.0 ± 12.8	130.1 ± 12.8*^	153.3 ± 18.4	149.7 ± 18.5
HbA_{1c} (%)	5.9 ± 0.4	5.5 ± 0.2*	5.8 ± 0.3	5.7 ± 0.2
FPI (μU/ml)	10.3 ± 6.7	9.2 ± 5.8*^	10.1 ± 6.5	10.5 ± 6.9
Homa index	2.80 ± 0.7	2.39 ± 0.4*^	2.84 ± 0.8	2.89 ± 0.9
TC (mg/dl)	215.1 ± 15.8	211.0 ± 14.2	218.6 ± 16.9	220.2 ± 18.1
LDL-C (mg/dl)	146.9 ± 18.4	143.4 ± 17.7	150.9 ± 19.2	152.6 ± 20.7
HDL-C (mg/dl)	43.8 ± 5.0	44.0 ± 5.1	43.6 ± 4.9	43.7 ± 4.8
Tg (mg/dl)	122.1 ± 24.2	117.3 ± 22.0	120.4 ± 23.5	119.5 ± 23.1
AST (UI/l)	18.8 ± 10.8	18.5 ± 10.4	18.2 ± 10.3	18.4 ± 10.5
ALT (UI/l)	28.3 ± 14.2	28.9 ± 14.5	26.8 ± 13.1	26.1 ± 12.8
γ-GT (UI/l)	24.5 ± 8.1	24.1 ± 7.7	25.8 ± 8.7	25.3 ± 8.4
Creatinine (mg/dl)	0.6 ± 0.2	0.7 ± 0.3	0.7 ± 0.3	0.8 ± 0.4

Hs-CRP (mg/l)	1.3 ± 0.5	1.0 ± 0.2*^	1.3 ± 0.5	1.4 ± 0.6

Table 2. Baseline, and 3-month anthropometric and biochemical parameters of patients during ABA containing nutraceutical (ABA) treatment and placebo.
Data are expressed as mean ± standard deviation. *p < 0.05 vs baseline; ^p < 0.05 vs placebo. M: males; F: females; BMI: body mass index; WC: waist circumference; HC: hip circumference; AC: abdominal circumference; FPG: fasting plasma glucose; PPG: postprandial plasma glucose; HbA_{1c}: glycated hemoglobin; FPI: fasting plasma insulin; HOMA index: homeostatic model assessment of insulin resistance; TC: total cholesterol; LDL-C: low density lipoprotein-cholesterol; HDL-C: high density lipoprotein-cholesterol; Tg: triglycerides; AST: aspartate aminotransferase; ALT: alanine aminotransferase; γ-GT: gamma-glutamyl transpeptidase; Hs-CRP: high sensitivity C-reactive protein.

3.2. Anthropometric parameters

No change was observed in BMI and circumferences in both treatments (Table 2).

3.3. Glyco-metabolic parameters

A significant decrease in FPG, PPG, FPI, and Homa index was observed in the ABA group compared to the baseline value ($p < 0.05$ vs baseline) and compared to the placebo group ($p < 0.05$ vs placebo). The HbA_{1c} value was significantly reduced compared to baseline ($p < 0.05$ vs baseline) in the group being treated with ABA (Table 2).

3.4. Lipid profile

No significant modification was observed in the lipid profile parameters, although a slight non-significant reduction was seen in TC, LDL-C and Tg in the ABA group (Table 2).

3.5. Inflammation parameter

Hs-CRP decreased significantly from baseline ($p < 0.05$ vs baseline) in the ABA group and also compared to the placebo group ($p < 0.05$ vs placebo) (Table 2).

3.6. OGTT results

At baseline, 39.4% of patients were affected by IFG in the ABA group vs 34.4% in placebo (p not significant), whilst 60.6% of patients were affected by IGT in the ABA group, and 65.6% in placebo group (p not significant). After 3 months, 26.7% of patients returned to a normal glycemic status in the ABA group vs 0 patients in placebo group ($p < 0.05$); at the end of the study, 20.0% were classified as IFG in the ABA group vs 33.3% in placebo group ($p < 0.05$). In the ABA group, 53.3% were classified as IGT vs 63.3 % in placebo group ($p < 0.01$). In placebo group, 6.7 % developed type 2 diabetes mellitus vs 0 patients in the ABA group (Table 1).

3.7. M value during euglycemic hyperinsulinemic clamp

Insulin sensitivity (M) value obtained after ABA treatment was higher respect to baseline ($p < 0.001$ vs baseline). No significant variations were recorded with placebo respect to baseline. Furthermore, M values recorded with ABA was higher than the one observed with placebo ($p < 0.0001$).

Considering as normal insulin sensitivity M value ≥ 7.5 mg (Kg min)$^{-1}$, at the end of the study, more patients returned to normal insulin sensitivity (89%) with the ABA treatment respect to placebo. Moreover, 11% of patients reached a M value ≥ 4 and < 7.5 mg (Kg min)$^{-1}$, and 0 patients had a M value < 4 mg (Kg min)$^{-1}$(value of insulin resistance) at the end of the study in the ABA treatment, respectively (Table 3)

	N	Baseline	End of treatment	Delta End of treatment vs Baseline
ABA	33	6.09 ± 0.51	7.38 ± 0.89*°	1.29 ± 0.59*
Placebo	32	6.02 ± 0.37	6.03 ± 0.76	0.01 ± 0.006

Table 3. M value variation during the study.
Data are expressed as SD: standard deviation. *p < 0.001 vs baseline; °p < 0.0001 vs placebo. Definition of insulin sensitivity: Normal insulin sensitivity: M value ≥ 7.5 mg (Kg min)$^{-1}$; Impaired glucose tolerance: M value ≥ 4 and < 7.5 mg (Kg min)$^{-1}$; Insulin resistance: M value < 4 mg (Kg min)$^{-1}$.

3.8. Glucagon test results

As expected, there was a significant increase of FPG and C-peptide after 6 minutes from the glucagon injection, at the baseline in ABA group, and in the placebo group (p< 0.01 for FPG and p< 0.001 for C-peptide vs time 0, respectively). At 3 months, the glucagon test was repeated and there was a significant increase in FPG at 6 minutes ($p < 0.01$ vs time 0), reduced compared to baseline ($p < 0.05$ vs baseline), but significant compared to the placebo group ($p < 0.05$ vs placebo) in the ABA group, while FPG and C-peptide were significantly increased at 6 minutes with an increase similar to that obtained with the same baseline glucagon test (p< 0.01 for FPG and p< 0.001 for C-peptide vs time 0, respectively) in the placebo group (Tables 4 and 5).

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	Baseline		3 months	
	Time 0	6 minutes	Time 0	6 minutes
FPG (mg/dl)	110.6 ± 7.3	148.6 ± 21.3^	103.8 ± 5.9*£	131.2 ± 16.1^*£
C-peptide (ng/ml)	7.15 ± 2.38	20.45 ± 7.59°	9.02 ± 4.72*£	31.07 ± 10.15°§§

Table 4. Glucagon test at the baseline, and after 3 months in ABA group
Data are expressed as mean ± standard deviations (SD). FPG: fasting plasma glucose. ^p< 0.01 vs time 0; °p< 0.001 vs time 0; *p< 0.05 vs baseline; $p< 0.01 vs baseline; £p< 0.05 vs Placebo; §p< 0.01 vs Placebo.

	Baseline		3 months	
	Time 0	**6 minutes**	**Time 0**	**6 minutes**
FPG (mg/dl)	111.5 ± 7.9	146.1 ± 20.2^	112.1 ± 8.4	148.5 ± 21.4^
C-peptide (ng/ml)	7.28 ± 2.51	21.33 ± 7.91°	7.02 ± 2.37	20.15 ± 6.88°

Table 5. Glucagon test at the baseline, and after 3 months in Placebo group.
Data are expressed as mean ± standard deviations (SD). FPG: fasting plasma glucose. ^p< 0.01 vs time 0; °p< 0.001 vs time 0.

3.9. Safety and treatment acceptance

No significant changes of transaminases, γ-GT or creatinine were recorded during the study. Considering a score among 1 and 10, where 1 is the worst, and 10 is the best, no differences were recorded between groups regarding acceptance of treatment that was well tolerated.

4. Discussion

The present study showed that ABA is effective in improving glyco-metabolic and inflammation parameters in patients with IFG or IGT.

Recently, Magnone et al. 2018 demonstrated that chronic consumption of a supplement containing low dose of ABA ameliorated the prediabetes markers (FPG -30.2% and HbA_{1c} -8.1%)in subjects with borderline values of FPG (≥ 100 mg dL^{-1}) and HbA_{1c} (≥ 5.7%) defined in the American Diabetes Association (ADA) recommendations for prediabetes (Magnone et al. 2018). It has been also proven that the improvement was greater in these subjects than in healthy ones, suggesting the beneficial effect of low dose ABA supplementation in prediabetics. The absence of increased insulin secretion was probably due to the stimulation by ABA of muscle glucose uptake and the greater sensitivity of human GLUT4-expressing cells to ABA than pancreatic β-cells.

Data are consistent with what reported by other authors results regarding a decrease in FPG of 4.9 mg dL^{-1} (-4.5%), in PPG of 13.9 mg dL^{-1} (-9.7%), in HbA_{1c} of 0.4% (-6.8%), in FPI of 1.1 μUmL^{-1} (-10.7%) and in Homa index of 0.41 (-14.6%) in prediabetic patients who assumed ABA.

Guri et al. in 2007 demonstrated, in obese and prediabetic mice, that the involvement of ABA in regulating glucose metabolism is due to its structural similarity to thiazolidinediones and its efficacy similar to that of these antidiabetic oral drugs (Guri et al., 2007).

Another study showed that in subjects in which ABA ameliorated prediabetes markers, its consumption also improved metabolic syndrome related parameters (BMI -4.5%, WC -9.0%, and TC -19.3%). These subjects

had borderline values of BMI (≥ 25 Kg m^{-2}), WC (≥ 88 cm in female and ≥ 102 cm in males) and TC (> 200 mg d L^{-1}) established by ATP III guidelines for metabolic syndrome (Stone el al. 2014).

In the present study the ABA treatment did not determine any change in anthropometric parameters as well as in lipid profile with the exception of a slight, but not significant, reduction in TC (-4.1 mg dL^{-1}, -1.9%), LDL-C (-3.5 mg dL^{-1}, -2.4%) and Tg (-4.8 mg dL^{-1}, -3.9%) which, however, is indicative of a downward trend.

ABA supplementation has been observed to significantly reduce Hs-CRP levels (-0.3 mg L^{-1}, -23.1%) thus improving the inflammatory status in overweight prediabetic patients. Diabetes and obesity share a low degree chronic systemic inflammation and insulin resistance. ABA intake has been demonstrated to reduce inflammation in obese and prediabetic mice. In this murine model ABA consumption at the lowest dose determined a decrease of FPG and insulin levels with an improvement of glucose tolerance. The histological analysis of white adipose tissue also showed a decrease of adipocytes hypertrophy and macrophages infiltration in adipose tissue in addition to a down-regulation of tumor necrosis factor-α (TNF-α) mRNA expression (Sturla et al. 2009).

Considering the euglycemic hyperinsulinemic clamp, the data observed in this study confirm that it is possible to improve insulin resistance. In fact, 36.7% of patients reported normal blood sugar (< 100 mg dL^{-1}) and an improvement in measured insulin sensitivity at the end of the study (see M value). None of patients treated with the formulated nutraceutical had a worsening of insulin resistance.

After the administration of glucagon, ABA gave a lower increase in FPG and a greater increase in C-peptide after 6 min compared to the values recorded during the glucagon stimulation test performed at the beginning of the study in the placebo group.The physiological mechanism explaining this circumstance could be attributed to the ABA stimulationof the glucose uptake in the muscle and increase of the sensitivity of human GLUT4-expressing cells and stimulates human pancreatic β-cells (Magnone et al. 2015).

Conclusion

Abscisic acid can be effective in ameliorating glyco-metabolic compensation and in reducing inflammatory status in patients with IFG or IGT. Overall, these results indicate that ABA, due to its glycemic-lowering activity, may play a pivotal role in physiology and in disorders of glucose homeostasis in humans.

A drawback of this result prompts the need to observe these changes over a longer period of time to verify the possible reversible effect after the treatment interruption. Another limiting key point could be the number of the patients involved in the trial hoping for trials with a higher number of patients. A final consideration is the limited, though statistically correct, number of patients in the study.

Chapter V

Lacto-Fermentation of tomatoes pomace with *Lactobacillus rhamnosus*and*Lactobacillus plantarum*enhance the activity *in vitro* and ameliorate the glucose levels *in vivo*

1. Introduction

Probiotics are nowadays considered as an adjuvant and complementary therapeutic agent for several health conditions, especially in metabolic and gastrointestinal disorders.The consumption of foods and beverages containing functional probiotic microorganisms is a growing, global consumer trend.Moreover, the lacto-fermentation carried out by microorganisms, such as probiotics, is often used as an important tool that can increase the amount of natural phytocompounds like polyphenols,due to enzyme release (Verbeker, 2005). *Lactobacillus* spp(LAB), are live microorganisms which if administered in enough amount in the daily diet, may result beneficial to the host (Salmerón et al. 2015). The idea which inspired this research is linked, to realize a probiotic juice, where acid lactic bacteria, were inoculated and growth in tomatoes pomace puree. Some of the applications of LAB include their use as antifungal and anti-mycotoxigenic agents, as described in the first part of this thesis, aid producers of functional food, probiotics and starter culture. After safety approval, the Food and Drug Administration (FDA) of the United States and the European Food Safety Agency (EFSA), numerous LAB species and food additives derived from them have been generally recognized as safe (GRAS). They have been proven to be effective in some diseases, in the present study their role in diabetes condition has been exploited. In animal models have been observed connections between an altered microbiota composition and the development of obesity, insulin resistance, and diabetes in the host through several mechanisms, such as altered fatty acid metabolism, modulation of gut peptide YY and glucagon-like peptide 1 secretion, activation of the lipopolysaccharide toll-like receptor-4 axis, and modulation of intestinal barrier integrity by glucagon-like peptide 2 (Sun and Buys, 2016). Moreover,current literature shows that *Lactobacillu*s sppstrains possess potent inhibitory activity against intestinal α amylase, suggesting anti-diabetic potential application. Keeping in mind the strong evidences along with their excellent tolerability profile,alacto-fermented formulation has been developed. The end point was to assess the impact between the bioactive compundscontent of tomatoes pomace(lycopene, polyphenols, etc.) and acid lactic bacteria in *vitro*and in *vivo*.

2. Materials and Method

2.1 Analytical material

HPLC-grade acetonitrile, acetone, methanol, dichloromethane and n-hexane were purchased from Sigma (Louis, Mo., United States). The solvents were filtered through a 0.45mm membrane filter (Millipore, Bedford, MA, USA) and then degassed in an ultrasonic bath before use. The polyphenols caffeic acid, ferulic acid, chlorogenic acid, vanilic acid, kaempferol, quercetin, myricetin, narigenin, apigenin, rutin. HPLC-grade methanol (MeOH) and HPLC-grade acetonitrile (ACN) were purchased from Sigma-Aldrich (Steinheim, Germany). β-carotene, lycopene, 2,2-diphenyl-1-picrylhydrazil (DPPH) were purchased from Sigma (St. Louis, Mo., United States). A Kinetex C18 (250 x 4.5mm) column Phenomenex® (Phenomenex®, Torrance, CA, United States) was used. Gallic acid and lycopene standards were obtained from (Sigma-Aldrich, St. Louis, MO, United States).

2.2 Microbial cultures preparation

0.5g of single strainsof*LactobacillusPlantarum*(Sofar, Trezzano Rosa, Italy), 0.5g of single stains of *LactobacillusRhamnosus*(Farmalabor, Canosa, Italy), and a mix of the two strains in a ratio 0.25:0.25 w/w have been used. The strains were reactivated using 15 mL of MRS (MRS, Oxoid, England) brothprepared by mixing 62 g in 1 L of H_2O, in sterile condition using a laminar flow hood equipped with a HEPA filter (Absolute Filters, Milano, Italy), and than left in an incubator at 37 °C for 24 h. All samples were centrifuged at 4000 rpm for five 5 min, washed twice with sterile in 0.85 % NaCl and PBS (phosphate buffer saline) reagents prepared in advanceusingautoclave at 120 °C under high pressure for 15 minutes. 2 mL of these cultures were inoculated for fermentation.

2.3 Sample collection and puree preparation

Tomato (*Solanum lycopersicum* Mill.) industrial processed pomace wastes (as mixture of skins and seeds) was obtained from La Torrente Company (Salerno, Italy) during the summer period, year 2018.
These tomato by-products were subsequently subjected to freeze drying and then stored at −40 °C before the analysis.
For preparing the puree, 1 g of tomatoes pomace was mixed with distilled water in ratio 1:20 w:V, and added with 1.5% glucose solution as a carbon source according to the procedure described by Salmeron et al. 2015. The initial pH was adjusted to 6.5 with Na_2CO_3.

2.4 Fermentation protocol

The two single *Lactobacillus* strains, and the mixed strains in a ratio of 1:1 (w:w) at a concentration of 10^6 CFU/mLwere added to tomatoes puree and incubated at 37 °C for 24 h and 48h. Then centrifuged at 4000 rpm for 10 minutes. Supernatants were collected and stored for the following analyses.The control puree was stored under the same conditions and the volume of the inoculum was replaced with the same volume of physiological solution (NaCl 8.5 gL^{-1}). This procedure was done in four separated test tubes which correspond totwo samples of single LAB and 1 mix LAB (1:1 w:w) and one control which was prepared without LAB. Samples have been identified with different codes, namely T1 to T4 as shown in Table 1.

T1 = Tomato pomace TP (control)
T2 = TP + *Lactobacillus Plantarum*
T3 = TP + *Lactobacillus Ramnosus*
T4 = TP + Lactobacillus Mix (1:1, w:w)

Table 1. Inoculation of samples scheme.

2.5 Determination of pH

The pH of fermented TPL was measured by a digital pH meter (Crison, Barcellona, Spain).

2.6 Sample preparation for HPLC analyses

Polyphenols were extracted in darkness, as previously described by Violoi et al. 2017 with slight modifications. An aliquot (1.0 g) of lyophilized tomatoes pomace was extracted with 20 mL ethanol (70%), stirred well for homogenization for one minute, then placed in a ultrasonic bath at 75 ± 2 °C, for 30 min, filtered with 0.45 μm filters and kept at -20 °C until analysis. The extraction was repeated three times.
To extract the lycopene the procedure described by Gómez-Romero et al. 2007 was followed with a few modifications. Three g oflyophilizedpomacewere added to 50 mL of a mixture dichloromethane:methanol (2:1, v/v) then homogenized in an Ultra-Turrax (IKA T25, Staufen, Germany) for 2 min, and sonicated in aicebathfor 15 min. The samples were then centrifuged at 6000 rmp for 10 min and the supernatants were collected. The extraction step was repeated another 2 times using further 50 mL of solvent until the extract had a very light pink color. The supernatants were evaporated to dryness in a rotary evaporator at 25 °C. The dried extract containing carotenoids were dissolved in 15 mL of dichloromethane and analyzed by HPLC-DAD.

To optimize extraction conditions butylated hydroxyanisole(BHA)has been added to the extraction solvent to prevent oxidative degradation.

2.7 HPLC-DAD Analyses

The tomatoes polyphenols were determined by HPLC/diode-array detector (DAD) analysis, performed using a HPLC system Jasco Extrema LC-4000 system (Jasco Inc., Easton, MD, USA) equipped with an auto sampler, a binary solvent pump, and a diode-array detector (DAD). The separation and quantification were achieved using a Synergy Polar-RP (150 x 4.6 mm) C18 column (Phenomenex, Torrance, CA, USA). The acquisition wavelength was set in the range of 200–400 nm. Mobile phase consisted 0.1% of formic acid (FA) in water (A), acetonitrile (B). Injection volume was 20 µL and flow rate was kept at 1 mL min^{-1}.

In the same equipment, the determination of lycopene were performed using a HPLC Jasco Extrema LC-4000 system (Jasco Inc., Easton, MD, USA) equipped with an auto sampler, a binary solvent pump, and a diode-array detector (DAD). A C30 (Phenomenex Torrance, CA, USA) column (4.6 × 250 mm, i.d.3 µm) at 35°C was used.The mobile phases consisted on methanol:dichlormethane:n-hexane (1:1:1 V/V/V) (phase A), acetonitrile (phase B). The multi-channel UV–vis detector was set at 247, 280, 320, and 450 nm. Retention time was 17 min. Duplicate separate analyses were carried out for each sample presented as mean ± SD.Different diluted lycopene standard solutions in a concentration range1-0.0001 mg mL^{-1} were prepared to set the calibration curve.

2.8 Biochemical analysis

Tomatoes puree samples were centrifuged at 5000 rmpfor 10min (Biofuge, Beckman, Los Angeles, CA, United States). The supernatant was filtered through 0.45 µm nylon syringe filter (Phenomenex ,Torrance, CA, United States) and used for biochemical analyses.

2.8.1 Antioxidant activity

The antioxidant activity of tomatoes puree was measured with respect to the radical scavenging ability of the antioxidants present in the sample using the stableradical 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich St. Louis, MO, USA). The analysis was performed by adding 50 µL of the tomato pomacesupernatant to 950 µL of a methanol solution of DPPH (153 mmol L^{-1}). The decrease in absorbance was determined with an UV-visible spectrophotometer (Beckman, Los Angeles, CA, USA). The absorbance of DPPH radical without antioxidant, the control, was measured. All determinations were done in triplicate. Inhibition was calculated according to the formula $[(Ai - Af /Ac)] \times 100$, where Ai is the absorbance of sample at t=0, Af is the absorbance after 6min, and Ac is the absorbance of the control at time zero (Brand-Williams et al, 1995).

2.8.2 Total Polyphenol Content

Total polyphenol content (TPC) was determined through Folin-Ciocalteau's method, using gallic acid as standard (Sigma-Aldrich St. Louis, MO, USA). In brief, 0.1 mL of samples (properly diluted with water in order to obtain an absorbance value within the linear range of the spectrophotometerwere added with 0.5 mL of Folin-Ciocalteau's (Sigma-Aldrich St. Louis, MO, USA) reagent and 0.2 mL of an aqueous solution of Na_2CO_3 (20%, w/V). The final volume was adjusted to 10 mL with water. After mixing, the samples were kept in the dark for 90 min. The absorbance was measured at 750 nm. Each sample was analyzed in triplicate and the concentration of total polyphenols was calculated in terms of gallic acid equivalents (GAE) (Di Lorenzo et al., 2015).

2.9 Enzymatic assay

2.9.1 Inhibition of α-amylase

The assay was performed according Dastjerdi et al.2015 with few modification. Different concentrations of tomatoes supernatants in range 50-250 μg mL^{-1} were dispersed in 1.0 mLof 2 M phosphate buffer (pH=6.9) and added with 0.5 mL of enzyme α-Amylase (4U mL^{-1}) and incubated at 37ºC for 10 min, followed by addition of 400 μL of starch 1% solution in all test tubes and incubated for 2h. After the incubation period, 2 mL of 3, 5-dinitrosalicylic acid (DNS) reagent were quickly added to all the samples and heated for 5 min. After cooling, the volume of the solution adjusted to 25.0 mL with distilled water and filtered. The α-amylase activity was determined at 540 nm usingaspectrophotometer(Beckman, Los Angeles, CA, USA).to measure absorbance. The same assay was performed also on the mix of the probiotic strains which were before grown at 37°C for 24 h in MRS broth. The supernatants obtained from the centrifuged culture was used to prepare a suspension at 10^5–10^6 CFU mL^{-1}.Acarbose at different concentration (1-0.001 ppm mL^{-1}) was used as a standard to prepare the calibration curve.The % inhibition was calculated according to the formula:

% inhibition= Abs control (540) -Abs control (540) / Abs control (540)

2.10 Preliminary test in vivo

2.10.1 Study treatments

According to the safety of natural product in dietary supplements and novel foods, as indicated by the updated version (January 2015) of Regulation (EC) No. 258/1997 of the European Commission, a preliminary test *in vivo* to evaluate in glucose metabolism the efficacy of tomatoes pomace and the tomatoes puree enriched with the *Lactobacillus* spp. as a prototype of functional food,has been performed in a small

group of healthy subjects (n=5). Since the functional food was developed to be tested for its hypoglycemic activity, anOral Glucose Tolerance Test (OGTT) was performed at t0, t30, t60, t90,t120 on human subjects.No general agreement has been reached regarding the concentration of probiotics necessary to achieve beneficial effects; usually counts from 10^6 to 10^8 CFUmL^{-1} are recommended (Saha 2001). In accordance with this guidelines, two treatment beverages (TBs) were prepared by mixing the glucose reference with the following samples: 2 g of tomato peels named (TP) and 2 g of tomato peels + 5 UFC 10^6 mL^{-1} of *LactobacillusPlantarum*and*LactobacillusRhamnosus*named TPF (tomatoes fermented puree).

2.10.2 Study procedures

The preliminary study was a multicentered trial conducted at the Samnium Medical Cooperative and the UCCP center (Benevento, Italy). Healthy patients between the ages of 18 and 70, were recruited in october 2020. Subjects were advised not to drink alcohol or to perform intense physical activity within 48 h before blood sampling. All blood samples were taken in the morning and collected in 10 mL EDTA tubes (Becton-Dickinson, Plymouth, UK) and the plasma was isolated by centrifugation (20 min, 2200 rpm, 4 °C). All samples were stored at -80 ° C until analysis. The values of the blood sugar were determinedusinga Free Carpe DiemDiacron®equipment(Diacron International S.r.l., Italy).

2.11 Statistical analysis

All experiments were carried out in triplicate, and each sample was analysed in duplicate. The results are expressed as mean ± standard deviation.

3. Results

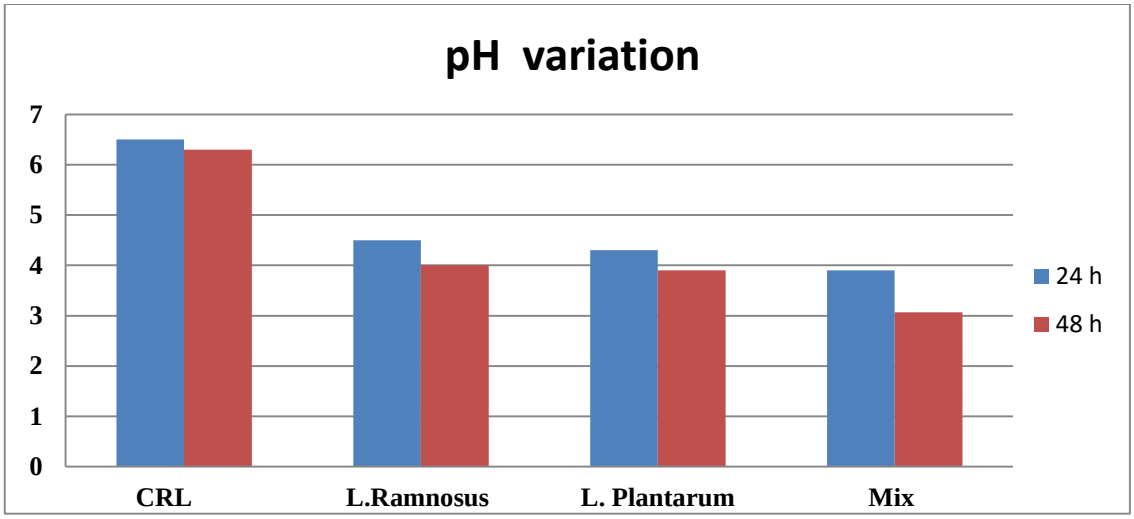
3.1 pH measurement

Two strains of acid lactic bacteria were used in our study: *L.Rhamnosus* and *L.Plantarum*. The concentrations were chosen according to the recommendation for probiotic foods (Verbeker et al. 2005). The pH values were measured at T0 (time of puree preparation) and after 24 and 48 h of incubation for both the strains tested. The initial pH of the tomato pomace was 6.5. The results revealed a linear decrease of the pH in both the formulations. After 24h-48h of fermentation the change in pH of the fermented samples varied from 4.41 ± 0.01 to 3.17 ± 0.02 between the strains. For the *L. Rhamnosus*, a decreases of 23% after 24 h of incubation at 37°C was observed and a decrease of 32 % after 48h of incubation. For the *L. Plantarum*, wasdetected a decrease of 22 % after 24 h of incubation at 37°C and of 30 % after 48h of incubation. The puree fermented by the mix of the *L. Plantarum* and *L. Rammnosus*in the ratio (1:1) showedthehighest reduction in pH (53%) compared to the strains and to the control as shown in Table 1 and Graphic 1. This indicates that both LAB strains have been efficient in the fermentation of tomato pomace. This allowed tospeculatethat the fermentative action can be beneficial of the synergy between the two strains.The decrease in pH is the indication of fermentation of the substrate and carbohydrate, an organic compound, is expected to yield organic acids such as lactic acid. The production of acid will increase the acidic content and thus reduction in pH value.Similarly, a rapid decrease in the pH has been observed due to fermentation of lactic acid bacteria (Zheng et al. 2015; Roja et al 2017; Yan et al 2019),which suggests that vegetable and fruit juices could be a good media for the probiotic growing.

Based on this, the mix of strains was used to evaluate *in vitro* the antioxidants, lycopene and polyphenols amount and the profile of glucose metabolism improvement *in vivo*.

Treatment details	**pH t=0h**	**pH t=24h**	**pHt= 48h**
T1 = Tomato pomace TP (Control)	6.5 ± 0.03	6.11 ± 0.02	5.81 ± 0.03
T2 = TP + *Lactobacillus Plantarum*	6.40 ±0.02	4.20 ± 0.01	3.61± 0.01
T3 = TP + *Lactobacillus Rammnosus*	6.40 ± 0.01	4.41 ± 0.01	3.67±0.02
T4 = TP + *Lactobacillus* Mix (1:1 p/p)	6.20 ± 0.01	4.05 ± 0.01	3.17±0.01

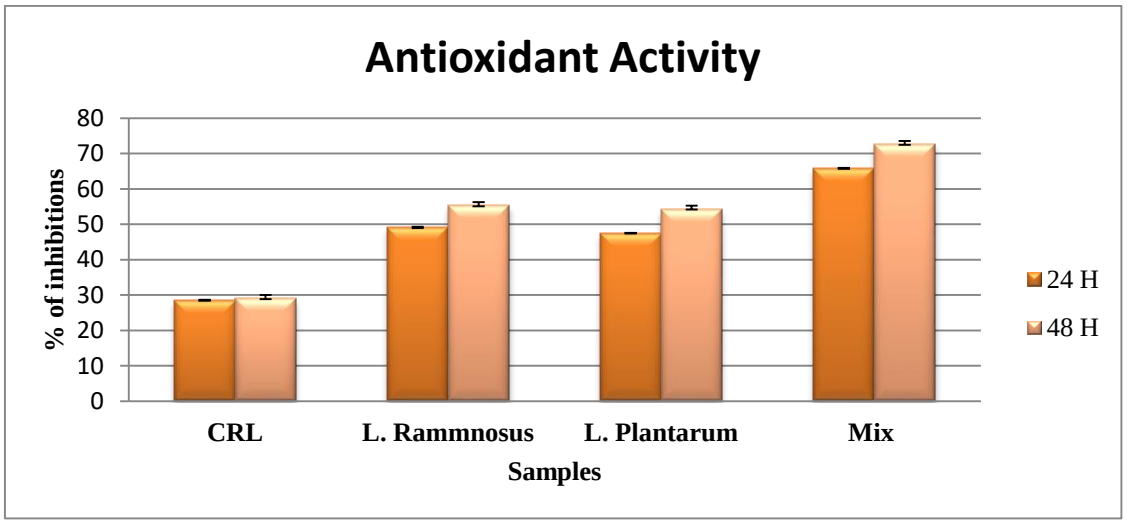
Table 1. pH measured after 24h and 48h. The analyses were performed in duplicate and the result are expressed as values ± standard deviation.



Graphic 1. Changes in pH of the fermented dried tomato pomace waste as influenced by different lactic acid bacteria.

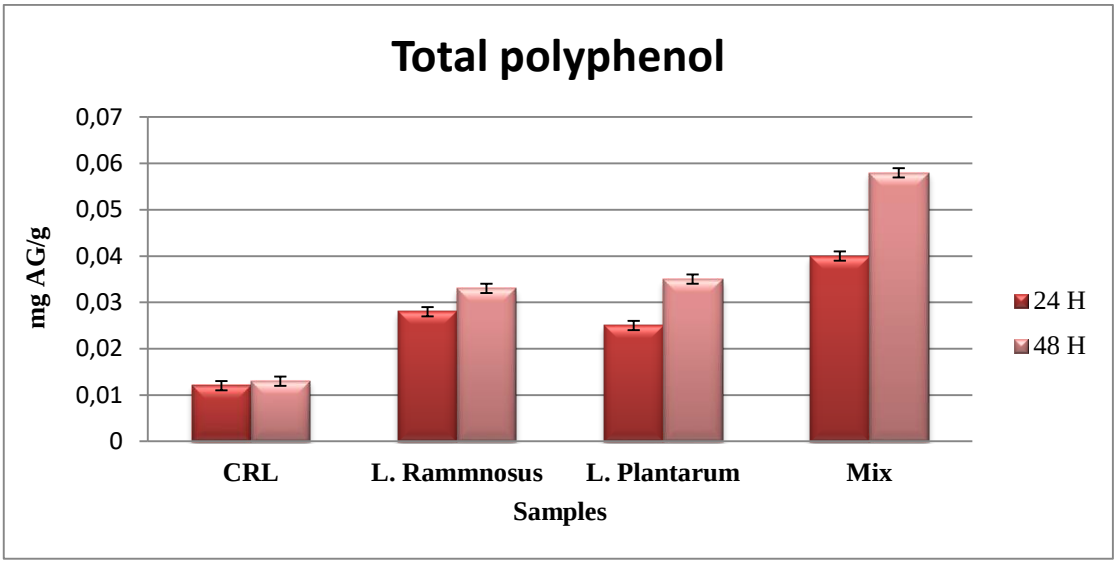
3.2 Antioxidant and total phenol content.

The capacity of strains to increase the bioaccesibility of phenolic compounds was evaluated by two different tests. The antioxidant activity of tomatoes puree was measured with respect to the radical scavenging ability of the antioxidants present in the sample using the stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) and the total polyphenol content (TPC) was determined through Folin-Ciocalteau's method. These tests were performed on the tomato puree supernatantwhere a possible enrichment of free phenolic compounds, released from the matrix after the probiotic fermentation, could have been observed. The data regarding the antioxidant activity and the total polyphenolic content are shown in the Graphics 2 and 3. The ability of fermentation to improve antioxidant activity is mainly due to an increase in the amount of phenolic compounds and flavonoids during fermentation, which is the result of a microbial hydrolysis reaction.



Graphic 2. Impact of the fermentation on the antioxidant activity. Increase of antioxidant activity was calculated against the control and expressed in % of inhibition.Values are reported as means ± SD (n =3).

The antioxidant properties in tomatoes pomace assessed by DPPH were markedly lower ($p < 0.05$) compared to fermented pomace. According to my results, the high % of inhibition,73.5 %, were obtained by the incubation of *Lactobacilli*mixat a concentration(5 x 10^6UFC mL^{-1}) of *L. plantarum* and *L. Rhamnosus*to be compared with the 28.59% of the control (non fermentedpureafter 48 h($p < 0.05$). No significant difference has been detected between the strains (p= 0.05) as shown in Graphic 2.Moreover, fermentation induces the structural breakdown of plant cell walls, leading to the releaseofantioxidant compounds amount. The antioxidant activity determined by DPHH assay positively correlated with the total polyphenol content as shown below. These antioxidant compounds can act as free radical terminators, metal chelators, singlet oxygen quenchers, or hydrogen donors to radicals.



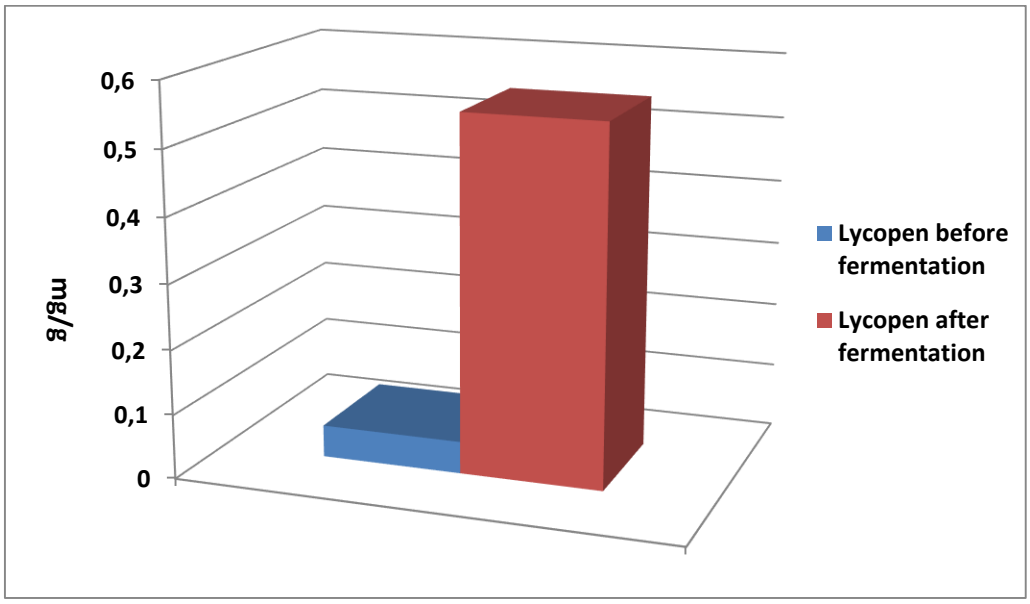
Graphic 3. Impact of the fermentation on the total polyphenol content.Increase of total polyphenols was calculated against the control and expressed in mg/g EGA (Gallic acid mg equivalent). Values are expressed as means ± SD (n = 3).

The efficiency of the fermentation process could be evaluated considering the phenolic compounds release. The results indicate that the fermentation by Lactobacilli have a positive impact on the increase of the free phenolic compounds. The optimum phenolic content was reached after 48 h of fermentation. In particular, *L. plantarum* and *L. Rhamnosus*gave total phenolic content of 35%-40%, respectively. These values can be expressed as 25.01 mg GAE g^{-1}dw and 28.4 mg GAE g^{-1}dw, respectively. No significant difference was observedbetweenthestrains (p = 0.48). On the opposite, the mix of the two strains released up to 85 % of the total phenolic compound compared to control (non fermented pure) (p< 0.05),expressed as 59.11 mg GAE g^{-1}dw. These result revealed that the mix has ability to breakdown the ester bonds composed in tomatos fiber liberating free phenolic compounds.

3.3 Bioactive compounds before and after the fermentation

3.3.1 Lycopene

The concentration of lycopene in tomatoes is limited since this compound is concentrated mainly within the intracellular components, such as the chromoplasts or the choloroplasts (Jamal *et al*., 2015). The extraction of lycopene has improved effectively under the fermentation. This may be due to the cellulase produced during this process, which is used to degrade the cell wall constituents, thus assisting the release of the intracellular contents (Ranveer et al., 2013). Lycopene can be absorbed by the human body after the releasefrom plant tissue structures into the gastrointestinal fluids. Alcoholic or lactic acid fermentations, as we have seen, can increase the release of lycopene. This is likely due to disruption of the plant tissue (Mapelli-Brahm et al, 2020). Data obtained by HPLC-DAD showed as how fermentation can increase the lycopene content (0.55 mg g^{-1}) by comparing the result with the control (extraction without fermentation) which was 0.05 mg g^{-1} as shown in Graphic 4.These results are in agreement with recent studies (Ejaz et al, 2018; Bartkiene et al, 2019), which suggested an increased release of lycopene after fermentation.



Graphic 4. Impact of the lactic fermentation on lycopene content.

3.3.2 Polyphenols

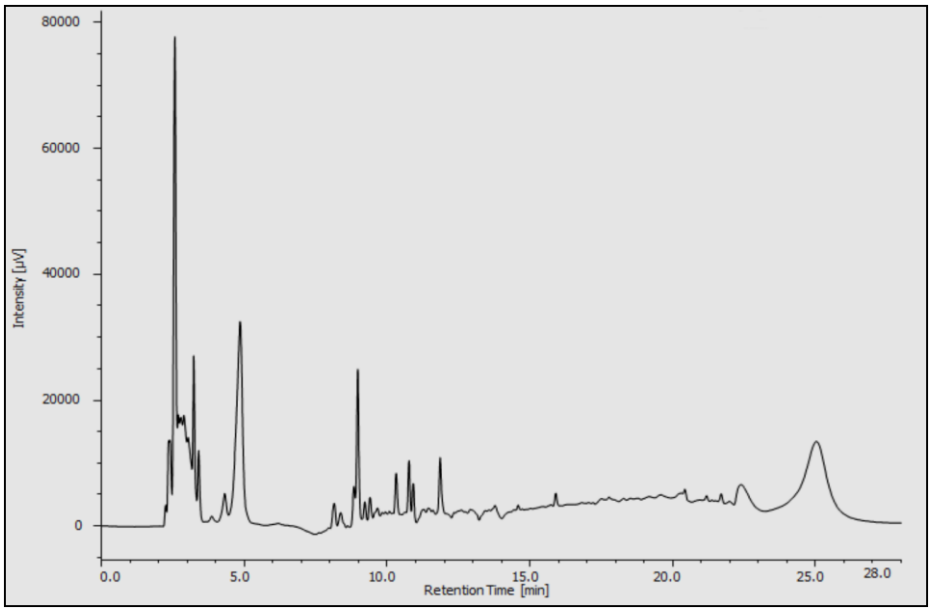
Polyphenols are the main natural antioxidants present in food. However, they are often bound to cell wall of fruit skin, glycosylated or in polymeric forms, which affect their bioaccessibility.Therelease of these bioactive compounds after fermentation by *Lactobacillus spp.* are reported by different authors (Bowey et al.2003; Bindon et al. 2010; Pereira et al. 2011).According to the results obtained from HPLC-DADin the present thesis work, rutin (quercetin 3-rhamnosylglucoside), the main polyphenol found in tomatoes with concentrations up to 0.2 mg g^{-1}dw, has been determined after the fermentation. This revealed an increased level of this compound by 57% as shown in chromatograms 1 and 2 below reported.

Chlorogenic acid is among the main polyphenolsbelonging to the hydroxycinnamic acid family. Its concentration has been determined as 0.05 ± 0.01 mg g^{-1}dw in the studied samples, in accordance with the concentration reported in the literature (Hubert et al. 2008; Martini et al 2016, Garzia-Valverde et al, 2013).

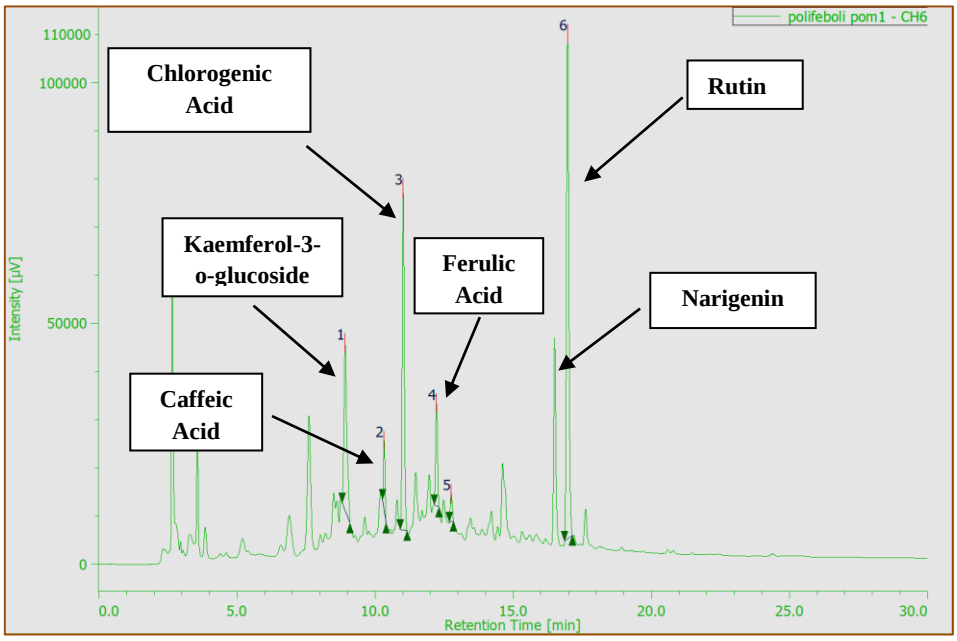
In general the main polyphenols increased their concentraton after the fermentation process (see Table 2). Enzymes produced during the fermentation, such as phenolic acid reductase and phenolic acid decarboxylase, contributed to polyphenol metabolismand may explain the increased levels of chlorogenic acid and other ploypheols in the analyzed samples. Moreover, glucosidase activity produced from *Lactobacillus spp*. was responsible for the reduction of flavonoids glycosides, which corresponded to the increase of the aglycones and naringenin. Table 2 summarize the polyphenol detected before and after fermentation in tomatoes pomace.

Polyphenol Content	**Before fermentation (mg g^{-1})**	**After fermentation (mg g^{-1})**
Rutin	0.21 ± 0.02	0.48 ± 0.01
Chlorogenic Acid	0.05 ± 0.01	0.25 ± 0.02
Caffeic Acid	0.003 ± 0.01	0.12 ± 0.01
Ferulic Acid	0.0085 ± 0.001	0.15 ± 0.02
Kaemferol-3-o-glucoside	0.02 ± 0.03	0.08 ±0.001
Narigenin	0.021 ± 0.01	0.16 ±0.02

Table 2. Main polyphenols identified in tomatoes pomace (mg g^{-1}) before and after fermentation. The analysis have been performed in triplicate and expressed as value ± SD.



Chromatogram 1: Polyphenol content before fermentation.



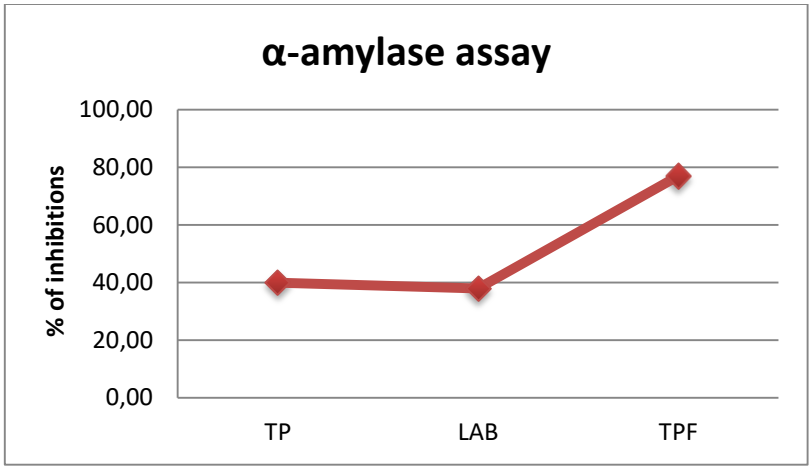
Chromatogram 2: Polyphenol content after fermentation.

3.4 Enzymatic assay in vitro

Alpha-amylase is responsible for postprandial glucose levels therefore, different plant extracts with α-amylase inhibitory activity are being investigated that might decrease postprandial blood glucose levels. The inhibition results of α-amylase for tomatoes pomace, mix of the LAB and the fermented pure are illustrated in Table 3 and Graphic 5 respectively. In the proposed study, a novelformulation*invitro*in order toevaluatecarbohydrate inhibition activity has been investigated. Acarbose has been used as positive control for theα-amylase inhibition. Each extract inhibited the activities of α-amylase in a dose-dependent manner. The inhibition percentage varied from 11.2 ± 0.1%to 77.2 ± 0.2 %.To quantify the inhibitory potential of the extracts, the half-maximal inhibitory concentration (IC_{50}) has been determined for each fraction that gave rise to 50% suppression of the original enzyme activity. On the basis of IC_{50} values, it was found that the fermented puree has significantly more potential for inhibiting these enzyme.Tomotoes puree fermented (TPF) exhibited α-amylase inhibition of 77.2% respect to control (tomatoes pomace) and the mix of the probiotic strains ($p < 0.05$).

Sample	Concentration mg mL^{-1}	% of Inhibition	IC_{50}
TP	50	12.8 ± 0.2	168.2 ± 0.12
	100	20.2 ± 0.4	
	150	29.1 ± 0.3	
	200	32.2 ± 0.4	
	250	**40.8 ± 0.1**	
LAB	50	11.2 ± 0.1	157.43 ±0.21
	100	19.9 ± 0.2	
	150	29.7 ± 0.3	
	200	30.2 ± 0.2	
	250	**39.9 ± 0.1**	
TPF	50	18.2 ± 0.1	102.1 ± 0.14
	100	29.2 ± 0.3	
	150	38.2 ± 0.2	
	200	52.2 ± 0.4	
	250	**77.2 ± 0.2**	

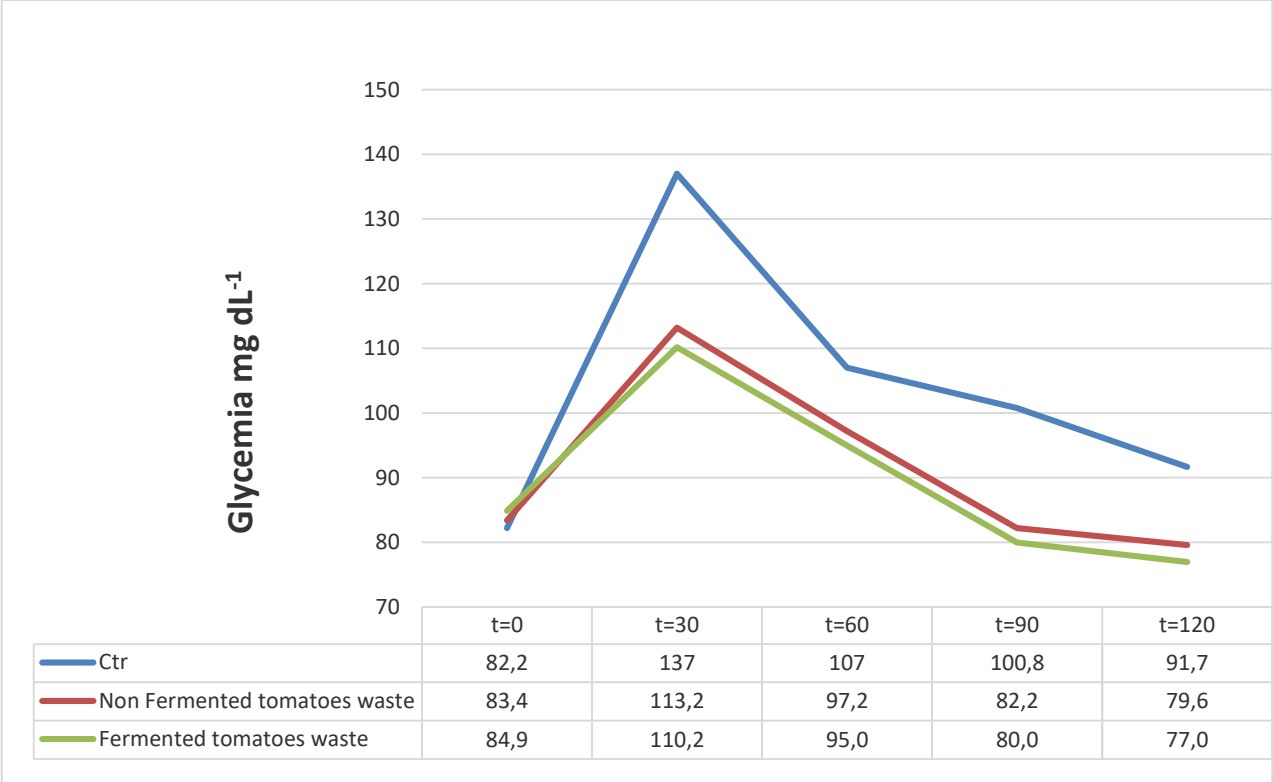
Table 3. α-amylase inhibitory activity *in vitro* of three different samples
All values are reported as the mean ± standard deviation of three replicates.



Graphic 5. Changes on % of inhibition of the enzyme alfa amylase from tree different samples. TP- Tomatoes pomace, LAB-*L. plantarum and L.Ramnosus*, TPF-Tomatoes pomace fermented.

3.5 Plasmatic glucose levels

As a follow up of the above mentioned data, apreliminary assessment has been performed on the glycemic response an oral glucose tolerance test (OGTT)*in vivo*.For the experimental assay, tomatoes pomace (TP) and Tomatoes Pomace Fermented (TPF) enriched with probiotics as a potential nutraceutical have been tested. A glucose reference solution (75% wV^{-1}) was administered and the individual's responses (n=5) wererecorded at at t0, t30, t60, t90, t120. The mean area under the curve (AUC) valuewassignificantly lower for both the formulations withrespect to the glucose reference solution (RG) $p<0.05$, (TP versus RG, 224 mg $dL^{-1}min^{-1}$ versus 278mg $dL^{-1}min^{-1}$ (p=0.02); TPF versus RG, 213 mg $dL^{-1}min^{-1}$vs278 mg $dL^{-1}min^{-1}$(p = 0.02). In particular, data obtained regarding the glycemic profile: i) showed a good activity as presented in Graphic 6; ii) demonstrated a decrease in blood glucose level at 60 and 90 min from the load, on average, from 107.0 mg dL^{-1} to 95.0 mgdL^{-1} and 100.8mg dL^{-1} to 80.0mg dL^{-1}respectively.This result opens the possibility to further studies to assess the efficacy of this novel nutraceutical.



	t=0	t=30	t=60	t=90	t=120
Ctr	82,2	137	107	100,8	91,7
Non Fermented tomatoes waste	83,4	113,2	97,2	82,2	79,6
Fermented tomatoes waste	84,9	110,2	95,0	80,0	77,0

Graphic 6. Impact of two formulations in glucose metabolism*in vivo* (n=5).
Results are espressed asmean±SD.

4. Discussion

Differentfoodsupplements, including antioxidant, vitamins, fibers, ω_3 fatty acids, probiotics, and herbs have been proposed for glycemic control.Tomatoes and *Lactobacillus spp.* have been shown to have functional and medicinal properties, but, to the best of our knowledge, none of the performed studies allowed a comprehensive *in vitro* and *in vivo*assessmenton the health promoting benefits of tomatoes pomace fermented with probiotics. Thepresented*in vitro* study showed that there is a decrease of pH values, which can be attributed to thesaccarolytic activity of *Lactobacillus* and to their ability to degrade the polysaccharides fiber and release short chain fatty acids like: butyrric, lactic, acetic acids, etc. (Nazzaro et al. 2009), which are responsible of the pH decrease. The novel formulation proposed in this thesis demonstrates the increasing of the total phenolic contents, lycopene and polyphenols and also the antioxidant capacity of tomatoes puree through microbial fermentation with lactic acid bacteria. Therefore, the inhibition of carbohydrate-hydrolyzing enzymes such as α-amylase decreasesthepostprandial hyperglycemia by retarding the glucose absorption. Many data on phenolics as potential inhibitors of thisenzymehave been reported

(Iwai et al. 2004; Williamson 2013),andin these regard,theresults obtained and described here could be attributed to the synergism of probiotic and phytocomplex (in particular the polyphenols). The clinical trial performed is only a preliminary screening, aimed to understand the real influence of our formulation on the glycemia metabolism. Although the patients enrolled didn't have glucose levels considered dangerous (<120 mg dL^{-1}). The most significative decrease was achieved after treatment with lacto-fermented tomatoes puree -63.8% to be compared with the tomatoes pomace-57.0%. A limiting key point could be the number of the patients involved in the trial, and the limited number of strains evaluated.

5. Conclusion

The data obtained from this research indicate that a diet enriched with nutraceuticalswhere probiotics are involved on a tomatoes waste matrix, may be a novel dietary supplement approach for the management of type 2 diabetes.The reported results may be useful for the development of nutraceuticals and beverages with improved health benefits, particularly in high blood sugar regulation and management of T2DM. Still further studies are needed in order to establish a better understanding of the molecular mechanisms of action which lead tobeneficialeffect on human health.

Capter VI

Closing remarks

Diabetes is a worldwidegrowing health issue, and nutraceuticals can be a challenge for the future in the area of prevention and/or therapy of this disease.Supporting the perspective of food as medicine, the aim of this Thesis has beento provide an innovative and safe alternative to pharmaceuticals through the determination of various phytochemical compounds (e.g. carotenoids, polyphenols, terpenoids, abscisic acid) present in threevegetal waste matrices, capable to prevent and/or to control diabetes.The major strengths of this thesis reside in its originality and in the evaluation*in vitro* and *in vivo* of the beneficial effects of three novel nutraceuticals formulated using nectarine thinning waste, tomatoes waste, olive leaves.The obtained results revealed that all the nutraceutical formulations were able to improve glycaemic and insulinemic responses, postprandial glycaemia (PPG), impaired fasting glucose (IFG), and impaired glucose tolerance (IGT).Another point developed in this Thesis has been to test theeffect of probiotics addition to tomato waste. This allowed to assess that the fermentation of this matrix produced a nutraceutical which is capable to improvetheglucose metabolism.All the positive results obtained can be useful to physicians to further developnutraceuticalbasedtreatmentsas a valuable support in the clinical practice.

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