Universidad Miguel Hernández

Escuela Politécnica Superior de Orihuela

Vegetable matrices as potential carriers for probiotic bacteria viability

Estefanía Valero Cases

Doctoral Thesis 2017

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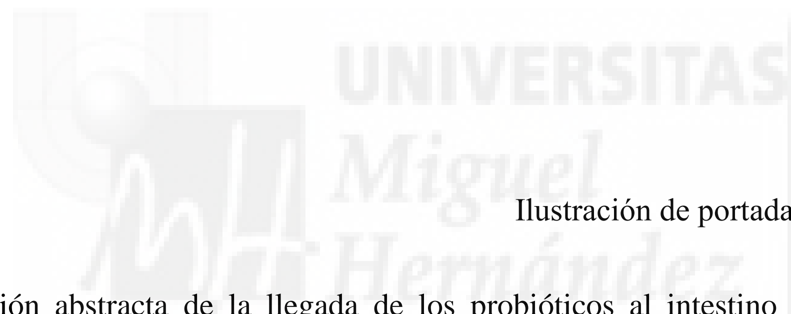

Ilustración de portada “*La llegada*”:

Representación abstracta de la llegada de los probióticos al intestino grueso que al encontrarse con las vellosidades intestinales (representadas en morado), aportan equilibrio e integración favoreciendo la homeostasis intestinal. Los colores de los microorganismos representan las matrices vegetales usadas como potenciales transportadores.

Por Estefanía Valero Cases

Escuela Politécnica Superior de Orihuela

Departamento de Tecnología Agroalimentaria

Vegetable matrices as potential carriers for probiotic bacteria viability

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TESIS DOCTORAL

Presentada por:

Estefanía Valero Cases

Directora:

Dra. María José Frutos

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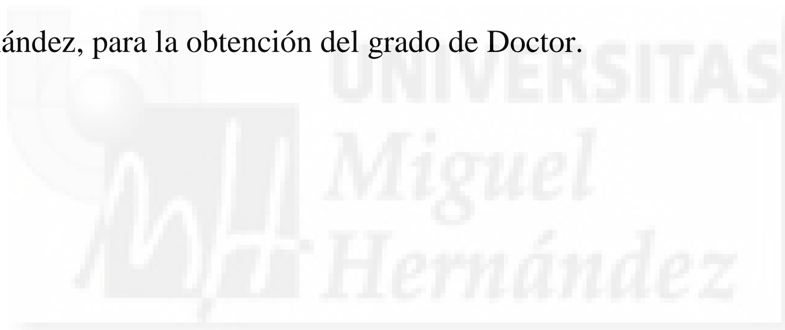
Vegetable matrices as potential carriers for probiotic bacteria viability

Tesis Doctoral realizada por Estefanía Valero Cases, Diplomada en Nutrición Humana y Dietética, Licenciada en Ciencia y Tecnología de los Alimentos y Máster en Biotecnología, en el Departamento de Tecnología Agroalimentaria de la Universidad Miguel Hernández, para la obtención del grado de Doctor.



Fdo.: Estefanía Valero Cases

Orihuela,________de____________de 2017

Dr. José Ramón Díaz Sánchez, Dr. Ingeniero Agrónomo, Catedrático de Escuela Universitaria y Director del Departamento de Tecnología Agroalimentaria de la Universidad Miguel Hernández,

CERTIFICA:

Que la Tesis Doctoral titulada "**Vegetable matrices as potential carriers for probiotic bacteria viability"** de la que es autora la Diplomada en Nutrición Humana y Dietética, Licenciada en Ciencia y Tecnología de los Alimentos y Máster en Biotecnología Estefanía Valero Cases ha sido realizada bajo la dirección de la Dra. María José Frutos Fernández, profesora Titular del Departamento de Tecnología Agroalimentaria; la considero conforme en cuanto a forma y contenido para que sea presentada para su correspondiente exposición pública.

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Dra. María José Frutos Fernández, profesora Titular del Departamento de Tecnología Agroalimentaria de la Universidad Miguel Hernández,

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Estefanía Valero Cases

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Doctoral Thesis structure

The Doctoral Thesis content has been prepared in agreement with the internal regulations of the Miguel Hernández University for the presentation of this Doctoral Thesis as a compendium of publications and for the European Doctor mention. Therefore, the structure of this Thesis is as follows:

- **Abstract** (in English and Spanish): where the overall summary is presented with a brief background, objectives and main results.
- **Introduction** (in English): it consists of a previous history of probiotics and prebiotics, definitions, criteria for both selections, as well as information on food vegetable matrices as probiotic carriers.
- **Objectives** (in English): the main and specific objectives of the research are detailed.
- **Publications**: where four published scientific articles, one scientific article under review and one national patent, are presented in the original language.
    - Productos gelificados probióticos o simbióticos y procedimiento para su obtención. ES2368401B2. (National Patent).
    - Effect of different types of encapsulation on the survival of *Lactobacillus plantarum* during storage with inulin and in vitro digestion. *LWT - Food Science and Technology, 64*(2), 824-828. doi: 10.1016/j.lwt.2015.06.049
    - Development of prebiotic nectars and juices as potential substrates for *Lactobacillus acidophilus*: Special reference to physicochemical characterization and consumer acceptability during storage. *LWT - Food Science and Technology, 81*, 136-143. doi: 10.1016/j.lwt.2017.03.047
    - Effect of Inulin on the Viability of *L. plantarum* during Storage and In Vitro Digestion and on Composition Parameters of Vegetable Fermented Juices. *Plant Foods for Humuman Nutrition*. doi: 10.1007/s11130-017-0601-x
    - Influence of Fermentation with Different Lactic Acid Bacteria and in Vitro Digestion on the Biotransformation of Phenolic Compounds in Fermented Pomegranate Juices. *Journal Agricultural and Food Chemistry*. doi: 10.1021/acs.jafc.6b04854
    - Influence of the Fruit Juices Carrier on the ability of *Lactobacillus plantarum* DSM20205 to improve in Vitro Intestinal Barrier Integrity and

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its Probiotic Properties. *Journal Agricultural and Food Chemistry* (under review).

- **Results and discussion** (in English): in this section, the most relevant results are analysed and discussed.
- **Conclusions** (in English and Spanish): the most relevant conclusions are listed.
- **References**: this section indicates the references used in sections complementary to the publications (Introduction, Results and Discussion).

NOTE: This manuscript does not include the section "Material and Methods" since they are described in the corresponding publications.

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Abstract

According to the Hippocrates´ aphorisms (460-370 BC) “Let food be your medicine and medicine be your food” and “All diseases begin in the gut”, nowadays, scientists studying the human microbiome suggest that healthy diets should include fermented foods to transiently strengthen living microbes in our gut. As a result, fermented food has gained popularity and consumers demand this type of food. However, most commercial probiotic foods in the market are dairy fermented foods and certain sectors of the population such as those allergic to milk proteins, strictly vegetarian and lactose intolerants, cannot consume them. Therefore, the need arises to explore new non-dairy matrices as carriers of probiotics to offer consumers an alternative to fermented dairy products. However, the use of probiotic cultures in alternative food matrices remains a critical problem because it could represent a major challenge for probiotic viability. Therefore, careful selection of food matrices as probiotic carriers is an essential factor in the development of probiotic foods to ensure a high viability of probiotics to reach the large intestine. Accordingly, the main objective of the PhD Thesis was to determine the influence of different vegetable matrices (polymeric matrices and beverages) as potential carriers for probiotic bacteria in order to ensure their viability in the range of 10^6-10^7 CFU/mL or g of food at the consumption time, to reach the large intestine in high amounts. The following aspects have been also investigated as part of the specific objectives: probiotic viability during manufacturing, storage and under gastrointestinal *in vitro* digestion, the synergistic effect of prebiotics and probiotics, the fermented beverages physicochemical parameters, antioxidant properties, sensory acceptance, and biotransformations of the phenolic compounds, and the influence of fruit juices as probiotic carriers on the ability of a probiotic strain to improve *in vitro* epithelial intestinal barrier integrity and the microbial intestinal adherence and potential cytotoxic effect to Caco-2 cells.

The results demonstrated that the probiotics studied were able to grow and survive during fermentation and manufacturing, remaining above the recommended concentrations during storage and under gastrointestinal *in vitro* digestion conditions in the different vegetable matrices. However, significant differences were observed in the probiotic viability in the polymeric and beverage matrices.

The growth, survival and the lactic acid production of the probiotic bacteria were strongly dependent of the chemical characteristics of the beverages; Inulin was metabolized by probiotics when the monosaccharides were at limited concentrations in the beverages during the fermentation and storage. The prebiotic effect of inulin on probiotic survival during *in vitro* gastrointestinal digestion was mainly observed after long periods of storage. The fermentation of vegetal beverages with different probiotic bacteria led to the biotransformation of phenolic compounds, suggesting a possible prebiotic effect of these phenolic compounds during the fermentation period and under the gastrointestinal *in vitro* digestion process. At the same time, the probiotic functional properties were strongly influenced by the different fermented matrices.

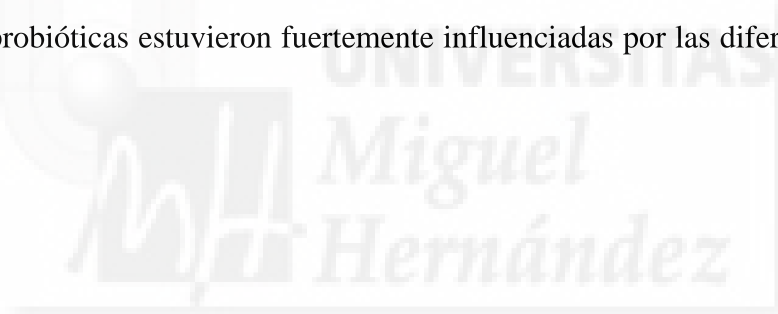
Resumen

Según los aforismos de Hipócrates (460-370 AD) "Que la medicina sea tu alimento y el alimento tu medicina" y "Todas las enfermedades comienzan en el intestino", actualmente, los científicos que estudian el microbioma humano aconsejan que una dieta saludable debe de incluir alimentos fermentados para mejorar de forma transitoria los microorganismos vivos en nuestro intestino. Como resultado, los alimentos fermentados han aumentado en popularidad y los consumidores demandan este tipo de alimentos. Sin embargo, la mayoría de alimentos probióticos que actualmente se encuentran en el mercado son de origen lácteo, los cuales no pueden ser consumidos por varios sectores de la población como: alérgicos a las proteínas lácteas, vegetarianos estrictos e intolerantes a la lactosa. Por lo tanto, existe la necesidad de investigar nuevas matrices alimentarias como vehículos de microorganismos probióticos para poder ofrecer una los consumidores una alternativa a los productos lácteos. Sin embargo, el uso de microorganismos probióticos en matrices alimentarias alternativas puede representar un reto para la viabilidad de los microorganismos. Por lo tanto, la cuidadosa selección de las matrices alimentarias como vehículos que puedan asegurar una elevada viabilidad de los probióticos para alcanzar el intestino grueso, es un factor esencial para el desarrollo de alimentos probióticos. Por consiguiente, el objetivo principal de la Tesis Doctoral, fue determinar la influencia de diferentes matrices vegetales (matrices poliméricas y bebidas) como potenciales transportadores de bacterias probióticas para poder asegurar su viabilidad en el rango de 10^6-10^7 UFC/mL o g de alimento en el momento del consumo, para poder alcanzar el intestino grueso en elevadas concentraciones. Como parte de los objetivos específicos se investigaron los siguientes aspectos: la viabilidad de los probióticos durante el proceso de elaboración, almacenamiento y bajo condiciones de digestión gastrointestinal *in vitro*, el efecto sinérgico de los prebióticos y probióticos, los parámetros fisicoquímicos de las bebidas, las propiedades antioxidantes, la aceptación sensorial, la biotransformación de los compuestos fenólicos de las matrices, así como la influencia de zumos de frutas como vehículos de los probióticos sobre la capacidad de los probióticos para mejorar la integridad de la barrera del epitelio intestinal, la adherencia microbiana y la citoxicidad a las células Caco-2.

Los resultados demostraron que los probióticos estudiados fueron capaces de crecer y sobrevivir durante la fermentación y los procesos de elaboración, permaneciendo por

encima de las concentraciones recomendadas durante el almacenamiento y bajo condiciones de digestión gastrointestinal *in vitro* en las diferentes matrices vegetales. Sin embargo, en las diferentes bebidas y matrices poliméricas, se encontraron diferencias significativas en la viabilidad de los probióticos.

El crecimiento, la supervivencia y la producción de ácido láctico de las bacterias probióticas estuvieron influenciados por las características químicas de las bebidas. Las bacterias probióticas metabolizaron la inulina durante la fermentación y almacenamiento, cuando las concentraciones de monosacáridos fueron escasas. El efecto prebiótico de la inulina sobre la supervivencia de los probióticos durante la digestión gastrointestinal *in vitro*, se observó principalmente tras largos periodos de almacenamiento. La fermentación de bebidas vegetales con diferentes bacterias probióticas indujo la biotransformación de los compuestos fenólicos, sugiriendo un posible efecto prebiótico de estos compuestos durante el periodo de fermentación y bajo condiciones de digestión gastrointestinal *in vitro*. Al mismo tiempo, las propiedades funcionales probióticas estuvieron fuertemente influenciadas por las diferentes matrices fermentadas.



INTRODUCTION

1. INTRODUCTION

1.1. Functional foods

Following the Hippocrates´ aphorism (460-370 BC) “Let food be your medicine and medicine be your food”, in recent years consumers are more aware of the relationship between diet and health and demand products with nutritional characteristics and specific components to prevent health issues and improve quality and life expectancy. This trend drives the growth of the functional food market and offers new product opportunities with specific components with health benefits beyond basic nutrition that meet consumer expectations (Ajmone-Marsan et al., 2014; Guerrero & Wilson, 2016; Villaño et al., 2016).

The concept of functional foods was born in Japan in 1980s, while Europe, Canada and the United States adopted this concept later. At present, functional foods are gaining prominence worldwide and the functional food market is expected to reach $ 192 billion in 2020 (Illanes & Guerrero, 2016; Kaur & Sing, 2017).

Nowadays, functional foods with different components have been developed to improve health benefits, such as bioactive compounds isolated from plants, probiotics, prebiotics, polyunsaturated fatty acids, vitamins and minerals among others (Gouw et al., 2017; Rathore et al., 2012; Vieira da Silva, 2016, Yasmin et al., 2015). Hippocrates also anticipated 25 centuries ago that "All diseases begin in the gut" by referring to the importance of the gastrointestinal system for human health. Nowadays, scientists studying the human microbiome suggest that healthy diets should include fermented foods to transiently strengthen living microbes in our gut. As a result, fermented foods have gained popularity and the consumers demand this type of food (Marco, et al., 2017; Plé et al., 2015). Probiotics and prebiotics are outstanding components of functional foods whose technological development in suitable matrices to ensure probiotic viability in high concentrations is the central theme of this doctoral thesis.

1.2. Probiotic bacteria

1.2.1. Fermentation and probiotics history

The probiotics history is part of human history because they are closely related to the use of fermented foods (Gasbarrini et al., 2016). Fermented foods and beverages have been elaborated and consumed for thousands of years and are now the staple foods of the human diet (Marco et al., 2017).

Before being aware of probiotic microorganisms, it is well documented that there was a wide variety of fermented foods depending on raw materials, environmental conditions and taste preferences that were widely used for nutritional and therapeutic purposes (Gogineni et al., 2013; Ozen & Dinleyici, 2015). The archaeological record shows evidences which suggest that the fermentation was known in Egypt and the Middle East (around 6000 BC) to make bread, beer, wine and fermented milk (McGovern, 2010). Legend tells that the nomadic towns travelling in Turkish desert carried the fresh milk in bags made from goatskin. The hot sun and the milk contact with the skin propitiated the multiplication of the acid bacteria and the milk became in a semisolid and tasty cream. This product was denominated yogurt (Gasbarrini et al., 2016). On the other hand, the first records of vegetables fermentation come from China around 300 BC; Chinese workers consumed fermented vegetables during the construction of Great Wall (Medina-Pradas et al., 2017). However, the role of microorganisms in fermentation processes was unknown for thousands of years, as the fermentation processes were made in an artisanal and traditional way which was extended from generation to generation (Caplice & Fitzgerald, 1999).

Awareness of the fermentation process and probiotic microorganisms began much later, when Louis Pasteur in 1861, identified the microorganisms responsible of the fermentation and their function in this process (Gasbarrini et al., 2016; Ross et al., 2002).

The term fermentation derived from the Latin *fervere,* to boil, and its meaning is related to the metabolic process by which a microorganism transform a carbohydrate (typically starch or a sugar) to generate a range of products that are mainly organic acids

(propionic, acetic and lactic acids), alcohol and carbon dioxide. The organic acids produced as the end of fermentation process, decrease the pH and provide an acid environment preventing the spoilage and extending the shelf-life of the fermented products (Gogineni et al., 2013; Ross et al., 2002; Stanbury et al., 2017).

However, Ilya Ilyich Metchnikoff in 1907 was the first scientific to find the beneficial relationship of lactic acid bacteria in the large intestine (**Figure 1**). His hypothesis was associated with the longevity of the Bulgarian population consuming sour milk fermented with *Lactobacillus bulgaricus* (previously denominated the *Bulgarian bacillus*) (Cavaillon & Legout, 2016). Metchnikoff also suggested that the ingestion of lactic acid bacteria promoted positive health effects by replacing the injurious effects of intestinal toxins from pathogenic bacterial (*proteolytic clostridium*) that contribute to aging and illness (Cavaillon & Legout, 2016; Gasbarrini et al., 2016). Therefore, Metchnikoff's researches were the first about probiotics but he did not develop the probiotic term.

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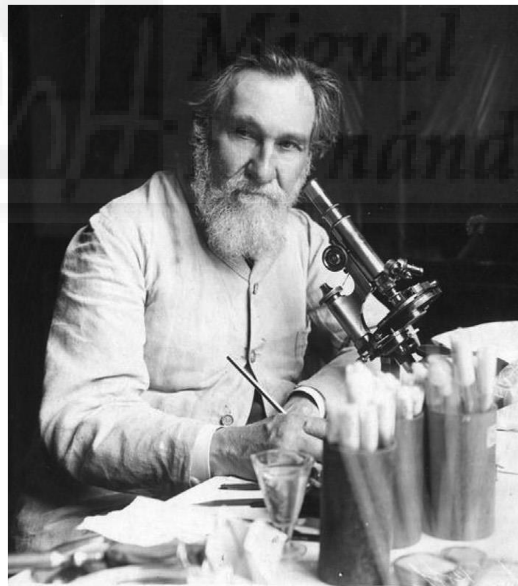

Figure 1. Ilya Ilyich Metchnikoff in his laboratory (Cavaillon & Legout, 2016)

However, nowadays, it is very important to point out that some fermented foods contain live microorganisms when consumed (eg. yogurt, kefir) while others (eg. beer, wine, fermented vegetables) are processed after fermentation (pasteurization, baking or filtering) and consequently they are not a source of living microorganisms.

1.2.2. Evolution of probiotics definition

The term probiotic means generally "for life" and derived from Latin (pro) and Greek (bios). However, over the last hundred years, following Metchnikoff's ideas, a lot of research has been carried out to support his hypothesis. In 1965, the probiotic term was defined for the first time by Lilly and Stillwell as "the anaerobic bacteria that are able to produce lactic acid and stimulate the growth of other microorganisms" (Lilly & Stillwell, 1965). Fuller (1989), suggested another probiotic definition as "live microbial feed supplements which beneficially affects the host animal by improving its intestinal microbial balance". However, in 2001, the expert work group of Food Agriculture Organization of United Nations (FAO) and World Health Organization (WHO) were the ones who defined the current and most widely probiotic definition as follows: "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (FAO/WHO, 2001).

1.2.3. The microorganisms mostly used as probiotics

The most popular bacteria used as probiotics in foods are the genera *Lactobacillus* and *Bifidobacterium* (**Table 1**). However, other genera as *Streptococcus*, *Leuconostoc* and *Enterococcus* are produced in powders or capsules more than used for food production (Abdollahi et al., 2016; Anadón et al., 2016; Butel, 2014).

Table 1. The main *Lactobacillus* and *Bifidobacterium* species used as probiotics

Genera	Species	
Lactobacillus	• *acidophilus* • *casei* • *fermentum* • *grasseri* • *johnsonii*	• *delbrueckii* • *rhamnosus* • *salivarius* • *plantarum* • *paracasei*
Bifidobacterium	• *longum* • *infantis* • *coagulans* • *breve*	• *bifidum* • *lactis* • *adolescentis*

Adapted from Illanes et al. (2016) and Sendra et al. (2016).

1.2.3.1. Lactobacillus genus

Lactobacillus belongs to the phylum *Firmicutes*, class *Bacilli* and order *Lactobacillales*, family *Lactobacillaceae*, genus *Lactobacillus*.

Lactobacillus genus includes a high number of Generally Recognized As Safe (GRAS)* species and is the most commonly used as probiotic and the most abundant regarding the described species (comprising 106) (Gaspar et al., 2013; Vasiljevic & Shah, 2008).

Lactobacillus can be found in vegetables, fruits, meat, dairy products, gastrointestinal and genital tract of humans and animals. They are Gram-positive bacteria, non-spore-forming and are predominantly catalase negative, they can appear as rods or coccobacilli generally characterized by low guanine-cytosine (G + C) content of the genome. They are facultative anaerobic bacteria and are generally mesophilic (30-40 ºC) but can grow at temperatures as high as 53 °C and as a low as 2 °C. Their optimum pH is in the range of 5.5 – 6.2, but can grow at pH 3 and 8 (Caplice & Fitzgerald, 1999).

Depending on their carbohydrate metabolism, *Lactobacillus* can be subdivided into two different groups with diverse fermentation end-products: homofermentative group (as obligate) and heterofermentative group (as facultative or obligate):

- The homofermentative *Lactobacillus* species generally ferment hexoses to produce lactic acid using pathway of Embden-Meyerhof-Parnas (EMP).

- The obligate heterofermentative *Lactobacillus* species ferment pentoses and hexoses to produce diverse end-products such as lactic acid, carbon dioxide, ethanol (or acetic acid) through the 6-phosphogluconate pathway (PP).

- The facultative heterofermentative *Lactobacillus* species use the EMP and PP pathways to ferment the hexoses and pentoses resulting in the production of etanol, acetic acid and formic acid under glucose limitation (Ross et al., 2002; Salvetti et al., 2013).

*GRAS is an American Food and Drug Administration (FDA) designation that a chemical or substance added to food is considered safe by experts, and so is exempted from the usual Federal Food, Drug, and Cosmetic Act (FFDCA) food additive tolerance requirements

On the other hand, the malolactic fermentation (MLF) is also carried out by *Lactobacillus*. The main aim of this kind of fermentation is the decarboxylation of malic acid to convert it into lactic acid. The MLF has an important influence in the development of aroma and flavour due to the production of secondary metabolites (such as diacetyl), mainly in wines (Boido et al., 2009; Sun et al., 2016).

1.2.3.2. Bifidobacterium genus

During a large period of the 20th century, the *Bifidobacterium* were classified as members of the genus *Lactobacillus* due to their similar physiological and morphological characteristics. However, both bacteria are gram-positive bacteria that differ in the content of G + C in their genomes: in bifidobacteria, the G + C content higher than *Lactobacillus* genera. The genus *Bifidobacterium* belongs to the phylum *Actinobacteria*, class *Actinobacteria*, sub-class *Actinobacteridae*, order *Bifidobacteriales* and *Bifidobacteriaceae* family (Felis & Dellaglio, 2007; Turroni et al., 2011).

Bifidobacterium are Gram-positive bacteria, non-spore-forming and are predominantly catalase negative, they may be present as polymorphic rods: from uniform to bifurcated in V and T rods (the most common) that have a protrusion at the end, such as a spatula or stick (Russell et al., 2011). These bacteria are obligate anaerobes and trough fructose-6-phosphete pathway (also known as bifid shunt) and ferment a variety of carbohydrates, (monosaccharides or disaccharides) to produce acid (but not gas) (Cronin et al., 2011; Felis & Dellaglio, 2007).

1.2.4. Criteria for selection of probiotic microorganisms

Some criteria must be considered for the probiotic selection, mainly the safety and stability, to remain physiologically active and genetically stable during production (Anadón et al., 2016; Illanes & Guerrero, 2016).

1.2.4.1. Safety and regulation criteria

The expert work group of FAO/WHO in 2002 published the guidelines for the assessment of probiotics in food. According to this guidelines, the microorganism should follow a testing process:

1. Identification the genus and species of the probiotic strain. The ability of probiotic bacteria to confer a health effect is strain specific. It is recommended that all strains be deposited in an internationally collection of culture.

2. *In vitro* test to screen potential probiotics, such as: gastric acidity and bile acid resistant, adherence to human epithelial cells and cells lines, antimicrobial activity against pathogenic bacteria, bile salt hydrolase activity.

3. Functionalized characterization and safety assessment test. According to these guidelines, the use of microorganisms for food fermentation must be Generally Recognized as Safe (GRAS). The evaluation of probiotic safety include: determination of antibiotic resistance patterns, potential virulence factor comprising toxicity and definite metabolic activities.

4. *In vivo* studies using animals and humans. It is significant to consider that the health benefits can be generated by different mechanisms: directly by the probiotic or through the production of probiotic metabolite(s) or enzyme(s) which act on the pathogens. Some examples of probiotic mechanisms on intestinal pathogens: production of antimicrobial substances, competitive exclusion of pathogen fixation, modulation of the immune system and competence for nutrients.

5. Health claim and labelling. The FAO/WHO working group recommended specific health claims and the information to be described on the label as follows: denomination of genus, species and strains and the minimum viable number of each probiotic strain at the end of the shelf-life. The food must provide the effective dose of probiotics related to the health claim(s), appropriate storage conditions and company contact details for consumer information.

The FAO/WHO documents (2001/2002) have been used by health and food safety agencies all over the world as references to elaborate their own guidelines for probiotics such as the European Food and Safety Authority (EFSA), the US Food and Drug Administration, Health Canada and Brazil, China, Argentina and Indian agencies, among others (Morelli, 2016). Respect to the microorganisms' identification, the European Union follows the consensus of the FAO/WHO (2002).

EFSA´s Panel on Biological Hazards (BIOHAZ) in 2007 (update 2013), has recently proposed a system "Qualified Presumption of Safety" (QPS) to ensure the microorganism safety previous to their commercialization (it is a similar concept and purpose of the GRAS definition used in the USA according to the FDA) (EFSA, 2013). This system is based on four basic concepts: the definition of taxonomic unit, knowledge body (history of use, scientific literature and databases, industrial applications, ecology and clinical aspects)), possible safety problems (pathogenicity) and the final provided use. The strains established as GRAS or its Europe equivalent, QPS, would allow to enter in the market without extensive efficacy and toxicity requirements (Anadón et al., 2016). The use of the strains deposited in an internationally recognized culture collection which followed the requirements according to FAO/WHO (2002) is also recommended (Sanz et al., 2016).

According to the European Union legislation on health claims (Regulation (EU) No 432/2012), communication of the health claims of probiotics strains to consumers can be made with prior authorization from the European Commission, which requires a favourable opinion from the EFSA´s panel on Dietetic Products, Nutrition and Allergies (NDA). Currently, the only health claim accepted by EFSA is for live yoghurt cultures (*Streptococcus salivarius* subsp. *Thermophilus* y *Lactobacillus delbrueckii* subsp. *Bulgaricus*): "Live cultures in yoghurt or fermented milk improve lactose digestion of the product in individuals who have difficulty digesting lactose". Therefore, the health claims are probably insufficient because of a problem of regulatory requirements more than a supporting evidence (Spinler et al., 2016). The International Probiotic Association (IPA) and International Scientific Association for Probiotics and Prebiotics (ISAPP) have been created to work with government bodies and industry to assist in establishing scientific standards for probiotics, generate high quality scientific information and struggle to change the existing confusion in the regulation of

probiotics. In October 2013, the ISAAP organized a meeting with panel expert group composed by members of the FAO/WHO Expert Panel, members of the FAO/WHO Working Group and other internationally recognized experts (Hill et al., 2013). The participants at the meeting jointly considered the key questions and approved some topics as:

- Include in the framework for the definition of probiotic microbial species that have been demonstrated in properly controlled studies to confer health benefits:

    - The use of the probiotic term for a species delivered in a functional dose for use as food or supplements for which systematic reviews or meta-analyses indicate a general benefit to the health of the population. For example, Health Canada has accepted the general health claim based on contribution to a healthy gut microbiota for following bacterial species: *Lactobacillus* (*acidophilus, casei, fermentum, gasseri, johnsonii, paracasei, plantarum, rhamnosus* and *salivarius*) and *Bifidobacterium* (*adolescentis, animalis, bifidum, breve* and *longum*) when delivered in food at a level of 1 x 10^9 CFU per serving. Similarly, the EFSA accepted characterization at the species and not strain level for health claim in yogurt cultures (Hill et al., 2013).

- Any specific claim beyond "contains probiotics" must be corroborated.

However, other specific effects at the intestinal or extra-intestinal level, including immune effect, are more likely to be strain-specific and health claims of such benefit can only be made for strains based on a scientific assessment of the evidence substantiating the claim. IPA Europe, is the European representation that take care of supporting its members and serving the general interests of the European probiotic industry. The IPA Europe affirms: “There is no harmonised EU legal framework establishing the conditions for a strain to be considered as probiotic or a positive list of individual strains which have a probiotic status”. Therefore, one of the main objectives is to establish channels of communication with the EU institutions to restore confidence in probiotics and to break the stagnation of probiotics at EU level (IPA Europe, 2015). In 2016, the EFSA published a new guideline on the scientific requirements for health

claims related to the immune system, the gastrointestinal tract and defence against pathogenic microorganisms that brings more clarity on the criteria to be considered to improve the probability to obtain a positive scientific opinion (EFSA, 2016).

1.2.4.2. Technological criteria

To select a potential probiotic strain is extremely important to consider the factors affecting probiotics survival, and that the strain resists the external conditions in the food product during manufacturing and storage, remaining viable in high concentrations of at least 10^6-10^7 colony forming units (CFU)/mL or g in foods at the consumption time. These amounts favour probiotic survival throughout gastrointestinal digestion to exert beneficial effect on the host (Rutella et al., 2016; Sánchez et al., 2016). However, many parameters can influence the probiotic viability in food products during production, processing and storage (Sendra et al., 2016; Tripathi, 2014):

- Microbial parameters:
  - Probiotic strain.
  - Preservation method of the strain (spray-dried, freeze-dried, frozen or encapsulated).
  - Proportion of inoculation.
- Food parameters:
  - Water activity.
  - Oxygen content.
  - pH and acidity.
  - Salt.
  - Sugars.
  - Artificial flavourings and colourings.
  - Preservation agents.
  - Microbial growth promoters (such as prebiotics, vitamins, minerals).
- Processing and storage parameters:
  - Heat treatment.
  - Cooling rate.
  - Incubation temperature.
  - Packaging materials.

- Storage temperature.

1.2.4.3. Functional criteria

The type and amount of bacteria varies throughout the different compartments of the gastrointestinal tract, only the large intestine contains around 10^{13}-10^{14} microorganisms (almost 10 times the number of the cells that form the human body and more than 100 times the genes contained in the body) equivalent to approximately 1.5 kg and most of them consist of bacteria phyla *Firmicutes* and *Bacteroidetes* (**Figure 2**). However, the intestinal microbiota composition is highly variable within individuals (Rupa & Mine, 2012; Tiihonene et al., 2010; Ventura et al., 2012). The development of the human intestinal microbiota begins at birth and may be influenced by factors such as: duration of gestation (preterm *vs* term), vaginal or caesarean delivery and by type of feeding (breast-fed or bottle-fed). The adult intestinal microbiota is established over the first 3-5 years and remains relatively stable throughout the life, but many times due to a poor diet and lifestyle (stress, depression, lack of physical activity, environmental factors, etc.) and prolonged consumption of medicines (such as in antibiotic treatments) a dysbiosis (pathogenic bacteria overgrowth) may occur in the intestinal microbiota by altering the intestinal motility and decreasing the integrity of the intestinal barrier. Moreover, the dysbiosis has been associated with the development of human diseases (obesity, diabetes, inflammatory bowel disease, irritable bowel syndrome, autism, multiple sclerosis, colon cancer) (Butel, 2014; Li et al., 2015; Vaiserman et al., 2017; Verdu et al., 2016;).

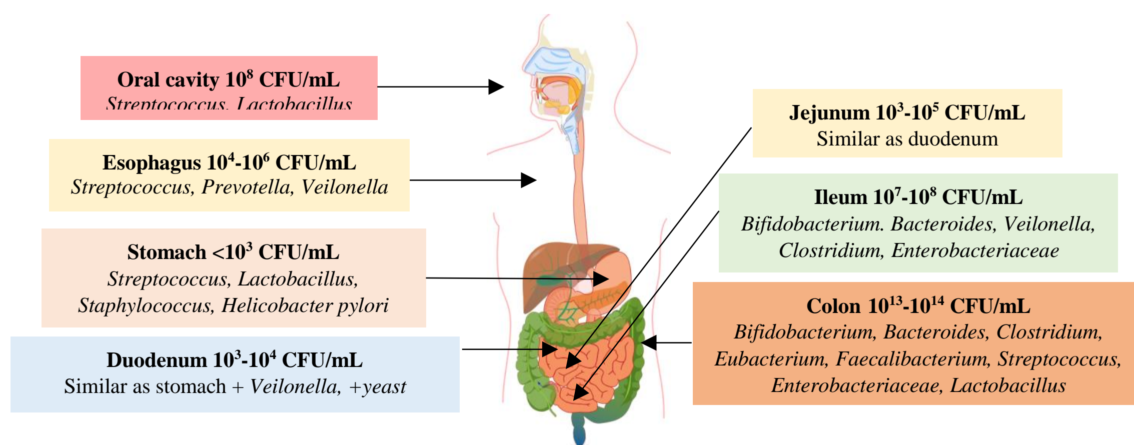

Figure 2. Level of bacteria in the different compartments of the gastrointestinal tract. Adapted from Holzapfel et al. (2001) and Tiihoenen et al. (2010).

The administration of live probiotics maintains the intestinal microbiota balance and contributes to the overall gut health. Probiotics can be consumed as part of fermented foods or through dietary supplements. To exert their beneficial effects, probiotics must survive to gastric acid and enzymes and adhere to the intestinal epithelium for an indefinite period of time (Tripathi & Giri, 2016). The survival and residence period of the probiotics in the colon can be influenced by the food matrix used to carry the probiotic, as well by the probiotic dose and duration (Ranadhera et al., 2012; Shori, 2016). The main health benefits provided by probiotics are shown below:

The probiotic fermentation of non-digestible carbohydrates results in short chain fatty acids (SCFA) and gases (CO_2, CH_4 and H_2) production that improve peristalsis. The SCFA consist in acetate (60%), propionate (25%) and butyrate (15%). The SCFA improve the intestinal barrier due to the drop in pH in the colon that inhibits the development and colonization of pathogens and thus, the production of toxic elements (such as ammonia) and the increase of mucus secretion and the growth of intestinal cells as colonocytes use the SCFA as main energy source (dos Reis et al., 2017; Rupa & Mine, 2012; Topping & Clifton 2001). At the same time, the SCFA are able to regulate the inflammatory reactions in the intestinal tract producing anti-inflammatory cytokines (such as interleukin (IL-10)) and inhibit the production of pro-inflammatory cytokines (IL-1β, IL-6, IL-8, IL-17, IL-12), and tumour necrosis factor-α (TNF-α) (immunomodulation) resulting in beneficial effects on inflammatory diseases and in the decrease of the risk for development colorectal cancer. Moreover, some probiotic microorganisms can produce antibacterial substances (bacteriocins, reuterin, hydrogen peroxide and lactic acid) that also inhibit the pathogenic bacteria growth (dos Reis et al., 2017; Richards et al., 2016). Lactic acid is a metabolite produced during fermentation by lactic acid bacteria and has recently been shown to regulate the pro-inflammatory cytokines production in intestinal epithelial cells in a dose-dependent manner (Iraporda et al., 2015).

Another mechanism of probiotic immunomodulation is the increased production of immunoglobulin A (Ig A) that contributes to biofilm formation in the gut to protect mucosal surfaces in continuous contact with toxic elements and pathogen microorganisms (Bollinger et al., 2003).The antibiotics use has resulted in an increase in antibiotic associate diarrhoea (ADD) and *Clostridium difficile* infection (CDD). In a

previous meta-analysis of randomised, double-blinded, placebo controlled trials, the patients involved treated with antibiotics were randomly assigned to probiotics administered (most of the probiotics used were *Lactobacillus* species) or to usual treatment, with or without placebo used for at least the duration of the antibiotic. The pooled results showed a significant reduction of the risk of CDD and ADD among the patients with randomly assigned probiotics (Hempel et al., 2012; Pattani et al., 2013; Videlock & Cremonini, 2012).

Recently many probiotic strains have demonstrated beneficial effects on the low density lipoproteins cholesterol (LDL) and hence having a beneficial effect through the coronary atherosclerosis reduction. Although the exact probiotic mechanisms for cholesterol reduction are still unclear, one of the pathways proposed is the cholesterol reduction through its attachment to the cell membrane because certain probiotic strains are able to produce exopolysaccharides (eg. five strain of *L. delbrueckii* subsp. *bulgaricus*) (Tok, 2010). Another pathway is that some probiotics (such as strains of *L. acidophilus*, *L. rhamnosus*, *L. plantarum B. adolescentis, B. longum*) have a bile salt hydrolase enzyme that catalyzes the deconjugation of bile salts and therefore, these salts are less hydrophilic and absorbable and can be excreted on the faeces in the free bile form (Kumar et al., 2012; Miremadi et al., 2014; Tok & Aslim, 2010).

1.3. Prebiotics

Prebiotic is a relatively new term, that was defined for the first time in 1995 by Gibson and Roberfroid as "a non-digestive food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health" (Gibson and Roberforid, 1995). However, during the las 20 years, this definition has been modified several times. In 2004, the same authors with other researchers, proposed a new version with slight changes: "a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers benefits upon host wellbeing and health" (Gibson et al., 2004). In both definitions the genera of target microorganisms for prebiotics are the *Lactobacillus* and *Bifidobacterium*. This new definition includes the wellbeing and specific changes in the whole gastrointestinal tract. In 2007, a new definition was proposed by the FAO: "A prebiotic is a non-viable

food component that confers a health benefits on the host associated with modulation of the microbiota" (FAO, 2007). However, this definition does not directly specify that the prebiotic is fermented by the intestinal microbiota. In 2010, Gibson et al., (2010), in disagreement with the FAO (2007) definition, emphasized "selectively fermentation" and proposed their ultimate definition (until now): "Dietary prebiotic: a selectively fermented ingredient that results in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health". The latest definition proposed was made recently by Bindels et al., (2015) that defined prebiotic as: "a non-digestible compound that, through its metabolism by microorganisms in the gut, modulates the composition and/or activity of the gut microbiota, thus conferring a beneficial physiological effect on the host". Although there is not a consensus definition so far, the key in any definition is that prebiotics should confer a beneficial effect on the host (Hutkins et al., 2016).

1.3.1. Sources of prebiotics and extraction methods

A range of products of dietary origin cannot be absorbed in the small intestine but can be metabolized in the colon by microflora through their fermentation. It is possible to get the prebiotics through a diet rich in fruits and vegetables (such as banana, garlic, asparagus or Jerusalem artichoke), but often the concentration that reaches the colon is not sufficient to exert the beneficial effect (Gibson et al., 2004). Therefore, it is necessary to obtain the prebiotics for their use as functional food ingredients. Nowadays, most of the prebiotic ingredients are non-digestible oligosaccharides, which have β-linkages on anomeric carbons that are resistant to hydrolysis human digestive enzymes being the opposite of digestible α-linkages (Sing et al., 2015; Wang, 2009). The most popular prebiotics, their extraction methods and their chemical identity are presented in the **Table 2.**

Table 2. Type, sources and extraction methods of prebiotics

Prebiotic	Sources	Chemical Identity	Extraction methods	References
Inulin	Jerusalem artichoke Chicory	Linear chains of D−fructose unit by β-(2→1) linkages D-fructofuranose with a terminal D−glucose linked to D−fructose by an α-(1→2) linkage. Chain length from 2 to 60. DP ≥ 10	Hot water	Li et al. (2015); Paseephol et al. (2007); Srinameb et al. (2015)
FOS	Agave Sucrose Chicory	Fructose units (range 2-4) unit by β-(2 → 1)-linked to a terminal glucose by an α-(2 → 1) linkage. DP from 2 to 10	1. Acid-catalysed hydrolysis 2. Transfructosylating process of β-fructosyltransferase or by β-fructofuranosidase 3. Enzymatic hydrolysis from inulin using endo-inulinases	Ávila-Fernández et at. (2011) ; Ganaie et al. (2013); Mussatto et al. (2013)
XOS	Corn cob, corn husk, cereal straw, sugarcane bagasse	Xylose units (range 2-6) through β-(1→4)-xylosidic linkages	1. Chemical method: • Acid hydrolysis (inorganic or organic acids) • Basic hydrolysis (NaOH or KOH) 2. Enzymatic hydrolysis. 3. Chemical and enzymatic combination.	Jayapal el al. (2013); Kumar & Satynarayana (2015); Samanta et al. (2012)
GOS	Lactose	Galactose units (range 2-5) with a terminal glucose linked together by different glycosidic linkages, being mostly β-(1→4) or β-(1→6)	Hydrolysis with β-galactosidase enzymes	Gonzáles-Delgado et al., (2016); Santibáñez et al., (2016); Urrutia (2013)
MOS	Starch	α-d-glucose units linked by α (1→4) glycosidic linkages	Debranching enzymes (pullulanase, and isoamylase) combined with different α-amylases	Rodríguez-Gascón et al. (2012)
IMO	Starch	Glucose unit (2-5) linked together by α-(1→6) linkages, with or without α-(1→4) linkage	Transglucosylation following enzymatic processes	Basu et al. (2016); Niu et al., (2017)
Lactulose	Lactose	4-O-β-d-galactopyranosyl-d-fructose Composed by of galactose and fructose	1. Chemical catalysis (alkaline medium) 2. Enzymatic catalysis (β-galactosidases)	Guerrero et al. (2011); Hajek et al. (2013); Song et al.(2013)

FOS:fructooligosaccharides; XOS: Xylooligosacchaarides; GOS: galactooligosaccharides; MOS: maltooligosaccharides. IMO: isomaltooligosaccharides

1.3.1.1. Inulin

Important criteria for the selection of the prebiotic source are that it should result in an increase in the beneficial bacteria but not having any limiting effect on the sensory attributes. Natural sources of inulin include chicory roots, Jerusalem artichoke, dahlia tubers, yacon, asparagus, leek, onion, banana, wheat and garlic. Inulin is a polysaccharide that consists of fructose units linked by β (2→1) linkages that are not hydrolyzed by human digestive enzymes. Therefore, inulin has much lower calorific value (4.184 kJ/g) respect to other carbohydrates, such as sucrose (16.736 kJ/g) (Gadekar et al., 2017). Inulin is used in a large number of technological food applications such as bakery products, increasing the fibre content, sweetener replacer (the sweetness level of inulin is about 10% of the sucrose), improving the textural and structural properties (Aidoo et al., 2014; Frutos et al., 2008; Mieszkowska & Marzec, 2016). Inulin has high level gelling properties (> 25% for standard chicory inulin and > 15% for long chain inulin) and may result in the formation of a creamy white structure which can be easily added in food as a fat substitute up to 100% (Akbari et al., 2016; Crispín-Isidro et al., 2015; Shoaib et al., 2016). Although the prebiotic activity of fructans has been widely studied as functional food ingredients, little information is available on the interaction between the fermentative capacity of the probiotic bacterial strain, the length of the fructan chain and the food matrix used (Karimi et al., 2015).

1.3.1.2. Polyphenols

Nowadays all compounds reported as probiotics are carbohydrates, however, previous prebiotic definitions do not say that prebiotics should be just carbohydrates. Recently, some research has been done to test other sources such as polyphenols as potential prebiotics (Steinert et al., 2016).

Polyphenols are part of our diet and exert antioxidant properties; the main dietary sources are wines, fruits, juices, legumes and vegetables (Lewandowska et al., 2013). The bioaccessibility of some polyphenols is very low because of their degree of polymerization and glycosylation pattern (Etxeberria et al., 2013). Therefore, their physiological benefits depend on the quantity of phenolic compounds that are available (bioavailability) to be absorbed in the intestine (Chandrasekara & Shahidi, 2012;

Fernandez-Garcia et al., 2009). This suggests that a large rate of phenolic compounds (as much as 90%) escape in the digestion in the upper gut and reach the colon and due to the action of gut microbiota. These compounds are transformed by some probiotic microbial enzymes, which can decompose the polyphenolic skeleton by dihydroxylation, demethylation and decarboxylation reactions resulting in relatively simple aromatic carboxylic acids, referred to as microbial phenolic acids metabolites, which can be absorbed to produce physiological effects (Etxeberria et al., 2013; Mosele et al., 2015). For example, the bioavailability of some flavonoids can be improved by fermentation of the flavonol glycosides by some probiotics with glucosidase activity, such as β-glucosidase, β-galactosidase or α-rhamnosidase (López de Lacey et al., 2014). However, there is little information on phenolic metabolites derived from microbial metabolism (Sadeghi Ekbatan et al., 2016; Tuohy et al., 2012). Many studies have shown the short chain fatty acids production from fermentable polyphenols (Parkar et al., 2013; Van Rymenant et al., 2017). However, the health effects are not the same for everyone because of different individual microbiota composition, so that the bioavailability of polyphenols for the production of microbial metabolites is subjected to interindividual variability (Etxeberria et al., 2013; Moco et al., 2012; Pandareesh et al., 2015).

In parallel to this microbial transformation in the colon, the microbial conversion of polyphenols can occur during a food fermentation process by lactic acid bacteria (Filannino et al., 2013; Filannino et al., 2015; Svensson et al., 2010). However, some factors must be considered such as the capacity of the lactic acid bacteria to metabolize the phenolic compounds depend on the species or on the strain and on the food matrix that may also influence the delayed release of phenolic compounds (Cueva et al., 2010; Filannino et al., 2015). Moreover, polyphenols are well known for their antioxidant activity; however, the antioxidant activity of the phenolic metabolites derived from microbial transformation has not been well studied (Sadeghi Ekbatan et al., 2016). Therefore, it is interesting to investigate the fermentation of food matrices rich in polyphenols, with different probiotics and to verify the possible production of metabolites and their antioxidant activity.

1.3.2. Criteria for selection of prebiotics

Gibson and Roberfroid (1995) published a prebiotic definition, together with a list of the necessary requirements to consider a food ingredient as a potential prebiotic:

- Resistance to gastrointestinal digestion (resistant to acidity, hydrolysis and absorption).
- Be a selective substrate that stimulates the growth and activity of one or more beneficial microorganisms.
- Be able to improve the beneficial microbiota in the colon that could be associated with the health benefits of the host.

Therefore, a prebiotic may be a fibre, but not all fibres may be a prebiotic (Samanta et al., 2015; Slavin, 2013).

1.3.2.1. Safety and regulation criteria

According to FAO Technical Meeting on prebiotics, the prebiotic ingredients should comply with the following safety parameters (FAO, 2007):

- Only when the prebiotic candidate has a safe use in the target host (such as GRAS or QPS) then it is suggested that further animal and human toxicological studies may not be necessary.
- Safe consumption levels with minimal symptoms and side effects should be established.
- The prebiotic candidate must not contain impurities and contaminants.
- The prebiotic should not alter the microbiota in such a way as to have long term detrimental effects on the host.

In Europe, EFSA uses prebiotic definition of FAO, which, as shown above, determines the need for beneficial effect on the host. Recently, the EFSA NDA panel gave a positive opinion to non-digestible carbohydrates and chicory inulin. The panel considers that the non-digestible carbohydrates result in a reduced post-prandial blood glucose

and insulinemic responses (EFSA, 2014). On the other hand, the panel considers that the consumption of native chicory inulin maintenances the normal bowel by increasing stool frequency (EFSA, 2015).

1.3.2.2. Technological criteria

According to Wang (2009), prebiotics must be chemically stable to the technological conditions of food processing such as:

- Heat treatment.
- Low pH.
- Maillard reaction.

At the same time, prebiotics have to offer the following technological advantages (Ávila-Fernández et al., 2011; Shoaib et al., 2016):

- Act as fat replacers due to their gelling properties
- Increase the viscosity and therefore the sensory properties such as mouthfeel and body.
- Act as sugar replacers due to their moderate sweetness.

1.3.2.3. Functional criteria

Among the beneficial effects produced by prebiotics the following may be noted (Al-sheraji et al., 2013):

- Improvement of the beneficial microbiota growth.
- Improvement of the bowel movement and regularity.
- Favourable influence on glucose and insulin levels.
- Production of SCFA thorough probiotic fermentation.
- Improvement of the mineral absorption.
- Blood lipid regulation.

To date, the FOS and inulin may have been the compounds most investigated as prebiotics (Meyer, 2015). But it is very important to consider the structure and degree of polymerization of prebiotics, since the prebiotic effect depends to a great extent on the probiotic ability to ferment them (Singh et al., 2015). Most of the prebiotic health benefits have been associated with probiotics. Synergistic action of probiotics and prebiotics is known as synbiotic and has been suggested to be more effective in terms of health benefits than probiotic and prebiotic actions separately (Finamore et al., 2016). Therefore, in order to improve the health benefits of the host, further research is needed to develop food products containing probiotics and prebiotics, known as synbiotic foods.

1.4. Vegetal matrices as probiotic carriers

Traditionally, dairy foods have been used as carriers of probiotic microorganisms. Therefore, dairy matrices with probiotic bacteria have been extensively explored in foods such as yogurts, milks, cheeses and kefir (Aljewicz & Cichosz, 2015; Mani-Lopez et al., 2014; O´Brien et al., 2016; Rutella et al., 2016). However, certain sectors of population may not consume dairy products such as: allergic to milk proteins, strictly vegetarian and lactose intolerant. Therefore, the need arises to explore new non-dairy matrices as carriers of probiotics to offer consumers an alternative to fermented milk products (Granado et al., 2010; Vijaya Kumar et al., 2015). However, the application of probiotic cultures in different food matrices remains a critical problem because it could represent a major challenge for probiotic viability. Moreover, the ideal food matrix should protect probiotic bacteria from the hostile medium during gastrointestinal digestion and deliver the probiotic at a high level to the main target organ, the large intestine (Capozzi et al., 2016; Shori, 2016).

Careful selection of the food matrix is an important factor to be considered in the development of probiotic foods, since the synergistic combination with prebiotic addition and the physicochemical and nutritional properties of the food matrices are important factors to ensure probiotic viability in the range of 10^6-10^7 CFU/mL or g of food at time of consumption. Another alternative to confer a high probiotic protection is the administration of microorganisms through polymer matrices (eg. microencapsulation). In this context, the formulated foods contain the microcapsules.

Thus, properly formulated food matrices can be an effective carrier of probiotics (Espita et al., 2016; Ranadheera et al., 2010).

1.4.1. Microencapsulation

Encapsulation is a mechanical or physicochemical process for retaining probiotics or bioactive food components (vitamins, antioxidants, fatty acids, minerals) in a matrix called a capsule with a size ranging from a few nanometres to a few millimetres (Solanki et al., 2013). Different microencapsulation methods have been developed to provide protection and improve the probiotic survival during manufacture, storage and during the gastrointestinal digestion process to reach the colon at high levels. However, some aspects should be considered to encapsulate probiotics by selecting the appropriate method of microencapsulation: (a) these microorganisms typically have a diameter between 1-5 μm, therefore nanotechnologies should be excluded, (b) they must remain alive when reaching the colon and (c) the capsules addition should not disturb the sensory properties (taste, texture or colour) of the food products (Burgain et al., 2011; Champagne & Fustier, 2007;). The **Table 3** shows the different techniques used to encapsulate probiotics.

1.4.1.1. Polymers used as encapsulating carriers

Selection of appropriate encapsulation carrier material is one of the main steps in the encapsulation process (Pang et al., 2014). The main criteria for selecting the encapsulation carrier material are that it must be: (a) generally recognized as safe (GRAS or QPS), biocompatible and biodegradable, (b) insoluble and resistant to ensure its integrity in both food and the upper gastrointestinal tract and (c) low cost (Nazzaro et al., 2012). The **Table 4** shows the advantages and limitations of main polymers used as encapsulating carriers.

Table 3. Encapsulation methods of probiotic bacteria.

Encapsulation	Technique	Capsules sizes	Encapsulation matrix	References
Extrusion	Using a syringe with a needle which contains a hydrocolloid solution (alginate) with the microorganism, and by extrusion the cell suspension is dropped over a hardening $CaCl_2$ solution.	Millimetre size to a few hundred microns. Depend on: • Needle diameter • Syringe Pressure • $[CaCl_2]$ • Stirring speed • Free fall distance	• Alginate • Carrageenan	Krasaekoopt et al. (2003); Nualkaekul el at. (2012)
Internal Emulsion	Water in oil emulsion with different steps: **1.** Microorganism + matrix polymer + insoluble calcium salt. **2.** Mixture 1 added into vegetable oil + surfactant. **3.** Organic acid to liberate Ca^{2+} for cross-linking with alginate.	≥100 µm Depend on: • Stirring speed • Water/oil ratio	• Alginate • Calcium salt: $CaCl_2$ or $CaCO_3$ • Surfactant:Tween® 80	Rodríguez-Llimós et al. (2003); Song et al. (2013)
External emulsion	**1.** Microorganism + matrix polymer + insoluble + vegetable oil. **2.** Calcium salt.	≥500 µm Possible agglomerates of microcapsules	• Alginate • Calcium salt: $CaCl_2$/ $CaCO_3$	Song et al. (2013)
Spray-drying	Probiotics are in solution that is atomized into a flow hot air (allowing the solvent to evaporate).	10-200 µm Possible agglomerates	• Starches • Gum Arabic • Maltodextrin	Alves et al. (2016); Peighambardoust et al. (2011)
Spray freeze drying	**1.** Probiotics are in solution that is atomized into a liquid nitrogen. **2.** Frozen droplets are dried by spray drying.	The particle size is more control than the spray-drying technique	Lactose, sucrose, cellulose	Burgain et al (2011); Her, Kim, and Lee (2015)
Coacervation	Probiotic solution is mixed with a matrix of an opposing charge, a complex is formed.	Depend on: • pH • [ion] • type and ratio of matrix	• Whey protein • Gelatin	de Vos et al. (2010)

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Table 4. Polymers used as probiotic bacteria carriers.

Polymer	Chemical identity	Advantages	Disadvantages	References
Alginate	Anionic and hydrophilic linear polysaccharide composed of 1,4′-linked β-D-mannuronic acid and α-L-guluronic acid residues obtained from marine algae.	• Low cost. • Compatible with almost all encapsulation techniques. • Resistant to gastric conditions, form gels in the presence of divalent cations, (Ca^{2+}).	• Porous. • Insoluble at low pH.	Solanaki et al. (2013)
Carrageenan	Anionic polysaccharide comprise alternating (1→3)-linked β-D-galactopyranosyl and (1→4)-linked α-D-galactopyranosyl units obtained from marine algae. There are three types κ- (kappa), ί- (iota), and λ- (lambda).	• κ- and ί- types form elastic gels in the presence of certain cations such as Ca^{2+} and K^{+} (in the form KCl used to induce gelation).	• High temperature to dissolve and melt. • KCl has an inhibitory effect on some LABs.	Kraseakoopt et al. (2003)
Agarose	Polysaccharide composed of β (1-3) linked D-galactose and α - (1→4) linked 3,6 -anhydro-L-galactose obtained from agar.	• Improvement of other polymer strength (gel)	• Porous. • High temperature to dissolve and melt.	de Vos et al. (2014); Mao et al. (2017)
Gelatine	Protein derived by partial hydrolysis of collagen (acidic or basic hydrolysis) of animal origin.	• Low cost. • Low fusion temperatures (30-40 ºC).	• Thermal reversible gel.	Comunian and Favaro-Trindade (2016); de Vos et al. (2014)
Chitosan	Cationic linear polysaccharide composed of 1,4′-linked glucosamine and N-acetyl glucosamine units obtained from deacetylation of chitin extracted from crustacean shells.	• Applied in combination with other polymers as shell.	• Solubility only at low pH. • It not used as capsule.	Martín et al. (2015); Solanaki et al. (2013)
Starch	Amylose is the linear constituent of starch, consisting of d-glucopyranose residues linked by α-(1-4) bond.	• The linkages are resistant to pancreatic α-amylase.	• The linkages are degraded by some probiotics.	de Vos et al. (2010)
Whey protein	mixtures of globular proteins (β-lactoglobulin (β-Lg), α-lactalbumin (α-La), blood serum albumin (BSA), lactoferrin (Lf), immunoglobulins (Ig).	• Resistant to proteolytic enzymes.	• Thermal treatments induce denaturation of bovine β-Lg.	Abd El-Salam and El-Shibiny (2016)

1.4.2. Vegetable probiotic beverages

Vegetable beverages are a good alternative to dairy probiotic food and a good choice for all ages, because they are refreshing, have attractive flavours, and furthermore, they are an excellent source of antioxidants, vitamins, bioactive compounds and minerals (Granado, et al., 2010; Kumar et al., 2015). The fermentation process can enhance the vegetables beverages shelf life and improve their nutritional properties with a final positive effect on human health (Shori, 2016). However, the probiotic viability in a vegetable matrix is more complicated than in dairy matrices and the physicochemical parameters need to be carefully controlled to ensure the probiotic viability, and that the sensory properties (flavour and aroma) that can remain or even be enhanced by the fermentation process (Marsh et al., 2014). In addition, probiotics must be resistant to processing and storage, remaining at high level (10^6-10^7 UFC/mL) at time of consumption to ensure their survival throughout gastrointestinal digestion and to be able to reach the colon in sufficient amount (Saarela et al., 2000).

1.5. Future perspectives

It is interesting to make further research in vegetable matrices as an alternative to fermented dairy foods. However, the success of new vegetable matrices depends on the probiotic ability to grow and survive in high amounts to provide sufficient viable cells to be able to beneficially modify the host´s intestinal microbiota (Shori, 2016). Therefore, as previously shown, for assessing a vegetable matrix as a potential carrier of probiotics, it is necessary to consider some aspects such as the technological criteria, processing and storage conditions. In addition, the probiotic survival during gastrointestinal digestion using different vegetable matrices that can ensure their protection should be investigated. Understanding the relationship between the food matrices and the probiotic bacteria is crucial for the development functional fermented foods.

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OBJETIVES

2. OBJECTIVES

2.1. Main objective

The main objective of this PhD Thesis was to determine the influence of different vegetable matrices as potential carriers for probiotic bacteria in order to ensure their viability in the range of 10^6-10^7 CFU/mL or g of food at the consumption time, to reach the large intestine in high amounts.

2.2. Specific objectives

- To develop probiotic or synbiotic gelled products and the method for their production ensuring a high microorganims viability during manufacturing and storage.

- To investigate the effect of different microencapsulation methods (extrusion and internal emulsion) and inulin on the viability of *Lactobacillus plantarum* CECT 220 during storage and *in vitro* digestion.

- To investigate the physicochemical parameters and the prebiotic effect of inulin in fermented carrot-orange juice on the *Lactobacillus plantarum* CECT 220 viability after fermentation during refrigerated storage and *in vitro* digestion.

- To investigate the effect of inulin supplementation on the viability of *Lactobacillus acidophilus* CECT 903, the physicochemical parameters and on the sensory acceptance during the storage of synbiotic nectars and juices.

- To investigate the antioxidant properties and the biotransformation of the phenolic compounds as potential prebiotics in pomegranate juices after fermentation by four lactic acid bacteria and during *in vitro* digestion.

- To investigate the influence of tomato and feijoa juices as fermentable carriers of *Lactobacillus plantarum* CECT 220 on the ability of this microorganism to improve *in vitro* intestinal barrier function using the trans-epithelial electrical resistance assay in an apical anaerobic model and its probiotic properties.

PUBLICATIONS

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Hernández

PUBLICATION 1

Productos gelificados probióticos o simbióticos y procedimiento para su obtención

María José Frutos, Estefanía Valero-Cases

PATENTE DE INVENCIÓN CON EXAMEN PREVIO ES2368401

UNIVERSITAS Miguel Hernández

PUBLICATION 2

Effect of different types of encapsulation on the survival of *Lactobacillus plantarum* during storage with inulin and *in vitro* digestion

Estefanía Valero-Cases, María José Frutos

LWT- Food Science and Technology 2015; 64, 824-828

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Effect of different types of encapsulation on the survival of *Lactobacillus plantarum* during storage with inulin and *in vitro* digestion

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ABSTRACT

The aim of this work was to investigate the effect of different microencapsulation methods (extrusion and internal emulsion microencapsulation) and inulin on the viability of *Lactobacillus plantarum* during storage at 4 °C. The inulin was added during microencapsulation at 0, 1 and 2%. The effect of the different phases of the gastrointestinal digestion on the survival of the microorganism during storage (0, 15 and 30 days) was also investigated. In both types of microcapsules, the best protection with higher survival of *Lactobacillus plantarum* during the 30 days of storage, was observed in the presence of 2% inulin with only 0.71 and 0.47 logs reduction for extrusion and emulsion microencapsulation respectively. From 15 days of storage the internal emulsion microcapsules did not maintain their structure during the *in vitro* digestion processes. At the end of the simulated gastrointestinal conditions (30 days), the number of cells were 7.40 and 6.53 log CFU g^{-1} for extrusion and emulsion microencapsulation respectively. In both microencapsulation methods, *Lactobacillus plantarum* showed a high survival ($\geq 10^6$ CFU g^{-1}). However for long storage periods, the best method for increasing the survival of *Lactobacillus plantarum* to the gastrointestinal digestion was the extrusion microencapsulation.

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1. Introduction

Probiotics are described by The Food and Agriculture Organization (FAO) of the United Nations and the World Health Organization (WHO) as "Live microorganisms (bacteria or yeasts), which when ingested or locally applied in sufficient number confer one or more specified demonstrated health benefits for the host" (FAO/WHO, 2001). To exert beneficial effects, the concentration of live microorganisms in the product should be above 10^6–10^7 CFU g^{-1} or mL^{-1} at the moment of consumption (FAO/WHO, 2001) and be able of survive to the digestive processes. The lactic acid bacteria (LAB) are the probiotic microorganisms more important linked to the human gastrointestinal tract. However their viability could be influenced by changes in temperature, pH, acidity, dissolved oxygen and hydrogen peroxide (Anal & Singh, 2007).

To provide the protection and survival of the LAB during storage and digestion, different microencapsulation methods have been developed, where probiotics are retained in a matrix that must be generally recognized as safe (GRAS) and insoluble to ensure its integrity in either the food and the upper part of the gastrointestinal tract (Krasaekoopt, Bhandari, & Deeth, 2003; Nazzaro, Orlando, Fratianni, & Coppola, 2012).

The material most widely used for the extrusion and emulsion microencapsulation techniques is the alginate, that is a natural polymer extracted from seaweed, consisting of 1 → 4 linked β-D-mannuronic and α-L-guluronic acids (Rinaudo, 2008). The extrusion microencapsulation technique is performed using a syringe with a needle which contains a hydrocolloid solution (alginate) with the microorganism, and by extrusion the cell suspension is dropped over a hardening $CaCl_2$ solution (Krasaekoopt et al., 2003). The emulsion microencapsulation originates microcapsules formed by the water in oil emulsion. The solution contains alginate, vegetable oil, calcium carbonate and a surfactant, and an organic acid that reacts with the calcium carbonate to form the microcapsules (Cook, Tzortzis, Charalampopoulos, & Khutoryanskiy, 2012; Krasaekoopt et al., 2003)

Prebiotics are described by Gibson, Probert, Loo, Rastall, and Roberfroid (2004) as "a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers benefits upon host well

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being and health". Inulin that is classified as soluble fiber, is one of the most known prebiotics. Is a reserve carbohydrate that is found in many fruits and vegetables as onion, garlic and banana among the most common ones (Flamm, Glinsmann, Kritchevsky, Prosky, & Roberfroid, 2001). This prebiotic reach the large intestine without modification and is available to be metabolized by microorganisms as *lactobacillus* and *bifidobacteria.* The combination of probiotics and prebiotics is known as symbiotic and is used in food products to take advantage of their synergic effects (Al-Sheraji et al., 2013).

The comparative studies of *in vitro* digestion among the different encapsulation methods during storage are scarce. The main aim of this work has been the study of the effect of two microencapsulation methods with different inulin concentrations on the survival of *Lactobacillus plantarum* during storage, and to test the influence of the *in vitro* digestion during that period on the release and survival of *L. plantarum*.

2. Material and methods

2.1. Bacterial strain and culture conditions

L. plantarum CECT 220 (ATCC 8014) was obtained from the Spanish Type Culture Collection (CECT, Valencia, Spain).The bacterial strain was prepared by growing the lyophilized culture in de Man Rogosa Sharpe (MRS) broth (Oxoid; Madrid, Spain) for 24 h at 37 °C under aerobic conditions to obtain an initial cell density about 10^8 Colony Forming Units per mL (CFU mL^{-1}). The cells were centrifuged at 2000 × g for 10 min at 4 °C, the pellet was washed sterile phosphate buffer saline (PBS) and was re-suspended in appropriate volume of PBS, resulting in a cell concentrate of about 9 log and were kept refrigerated 4 °C.

2.2. Microencapsulation of L. plantarum

2.2.1. Extrusion microencapsulation (EM)

The microencapsulation was made according to the method described by Nazzaro, Fratianni, Coppola, Sada, and Orlando (2009). Solutions were made with 2% Na-alginate, 5.5% MRS broth, 5% glycerol, 0.15% xanthan gum and with different amounts of the short chain artichoke inulin with a degree of polymerization 10 (Farma-química; Málaga, Spain), was added 0% (EM), 1% (EMI1%) and 2% (EMI2%), and were sterilized at 121 °C for 15 min. The solutions were cooled at 25 °C and *L. plantarum* was added at a concentration of 1:10 (microbial culture: alginate solution). Suspension was dropped through a 21G needle into sterile 0.05 M $CaCl_2$ (Scharlau; Barcelona, Spain) and was allowed to harden for 30 min. The microcapsules were washed with sterile NaCl solution at 0.9%, immersed in aseptic ultrapure water and stored at 4 °C.

2.2.2. Internal emulsion microencapsulation (IM)

The internal emulsion microencapsulation was made according to the method of Rodriquez-LLimos, Chiappetta, Szelig, Fernández, and Bregni (2003) with modifications: The Na-alginate solution was prepared following the same procedure as for extrusion microencapsulation. Inulin was also added at different proportions: 0% (IM), 1% (IMI1%) and 2% (IMI2%).

L. plantarum was added at a concentration of 1:10 (microbial culture:Na-alginate solution) under continuous magnetic stirring at 500 rpm together with 0.4% of $CaCO_3$ (Panreac; Barcelona, Spain). The emulsion was heated at 45 °C and 200 mL of olive oil and 4 mL Tween 80 (Panreac; Barcelona, Spain) were added at 45 °C under magnetic stirring at 500 rpm for 15 min to obtain the water/oil emulsion. For the internal ionic gelation, 1.7 mL of glacial acetic acid (Panreac; Barcelona, Spain) was added and stirred for 15 min at 500 rpm. The microcapsules formed were washed with a sterile NaCl solution at 0.9% and stored in aseptic ultrapure water at 4 °C.

2.3. Size and structure of microcapsules

The diameters of 100 microcapsules for each treatment were measured using an electric digital micrometer (Insize IP65, Spain). The results were expressed in mm.

The shape and surface of the microcapsules before and after digestion, was observed with a stereomicroscope Leica MZ95 (Leica, Spain)

2.4. Bacterial enumeration and survival during storage

The survival of *L. plantarum* microencapsulated with different amounts of inulin was evaluated during 30 days of storage at 4 °C. Samples were taken at 0, 1, 8, 15 and 30 days. The bacterial count was made with 1 g of each microencapsulated were blended in a stomacher with 9 mL of sterile peptone water for 10 min to dissolve. The samples were serially diluted into 0.1% peptone water and 0.1 mL was spread plated on MRS agar under aerobic conditions at 37 °C for 48 h. The results were expressed as log CFU g^{-1} of microcapsules.

2.5. Survival of microencapsulated L. plantarum to simulated gastrointestinal conditions

For the study of the storage times (0, 15 and 30 days) of the microencapsulated samples on the survival during the gastrointestinal digestion lasting 60 min. The EM and IM microcapsules were made using higher *L. plantarum* concentrations of 0.5:1 (culture solution:Na-alginate solution). The simulated gastrointestinal digestion of the microencapsulated *L. plantarum* was made according to previously described methods (Anal & Singh, 2007; Nazzaro et al., 2009)

The simulated gastric juices (SGJ) were prepared with MRS broth (Oxoid; Madrid, Spain) and 3 g/L of pepsin (Farma-química; Málaga, Spain). The pH was adjusted to 3 with HCl 0.1N (Panreac; Barcelona, Spain). The simulated intestinal juice (SIJ), was prepared with 1 g/L of pancreatin (Sigma; Madrid, Spain) and 4.5 g/L of bile salts (Sigma; Madrid, Spain). The pH of MRS broth was adjusted to pH 7 with NaOH 0.1 N (Panreac; Barcelona, Spain). Both solutions were sterile-filtered through a membrane (0.45 μm, Millipore; Spain).

The microcapsules (1 g) were homogenized for 2 min in a vortex (Selecta; Barcelona, Spain) with 9 mL of SJG at 37 °C and were incubated during 60 min at 37 °C. The enzymatic reaction was stopped by neutralization with 1 N NaOH, to pH 7. The SIJ (9 mL) and MRS broth were then added to the suspension up to a volume of 20 and was incubated for 60 min at 37 °C. The viable count in both SGJ and SIJ was determined by the plate count method in MRS agar and expressed as log CFU g^{-1}.

2.6. Statistical analysis

All experiments and analysis were made in triplicate. The results were expressed as mean ± standard deviation. The analysis of variance followed by a Duncan test ($p < 0.05$) was used for the mean comparison, using SPSS v21.0 software package (SPSS Inc., Chicago-Illinois-USA).

3. Results and discussion

3.1. Size and structure of probiotic beads

Fig. 1 shows the average sizes obtained for each of the types of the beads. There was a big variation in beads size depending on

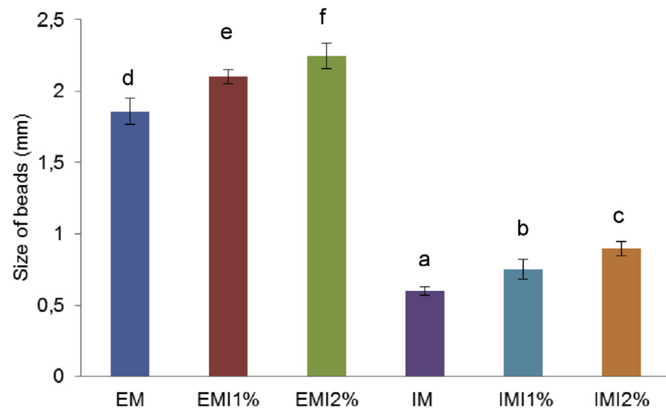

Fig. 1. Different sizes of microcapsules (mm) expressed as mean of 100 microcapsules± standard deviation. Bars with different lowercase letters are significantly ($p < 0.05$) different. EM: control extrusion microencapsulation; EMI1%: extrusion microencapsulation with 1% of inulin; EMI2%: extrusion microencapsulation with 2% of inulin; IM: control internal emulsion microencapsulation; IMI1%: internal emulsion microencapsulation with 1% of inulin; IMI2%: internal emulsion microencapsulation with 2% of inulin.

the methods of encapsulation and on the prebiotic content. As it can be observed, the size of the beads obtained by extrusion was higher and more homogeneous than those obtained by IM, with sizes between 1.86 mm and 2.25 mm. However in the IM, the size was much lower and with irregular shape, with values between 0.6 mm and 0.9 mm. The addition of different concentrations of inulin increased significantly ($p < 0.05$) the size of the beads. The capsules with 2% of inulin were significantly higher than those with 1% of inulin and without inulin in both microencapsulation methods. The beads of EM 2% were presented the largest size and the beads of IM were the smaller one. Chavarri et al. (2010); Krasaekoopt and Watcharapoka (2014) also reported that addition of quercetin and different concentrations of prebiotics (GOS and inulin) increased the size of the capsules obtained with extrusion microencapsulation.

However, the size of the microcapsules in the EM also could be influenced by the alginate concentration, the diameter of the needle, the pressure on the syringe, the $CaCl_2$ concentration and the stirring speed in the solution where the alginate was dropped to form the capsules. Nualkaekul, Lenton, Cook, Khutoryanskiy, and Charalampopoulos (2012) obtained an average size of 2.9 mm for the extrusion microencapsulation without coating that was higher than the average size obtained in our study for all the beads. However, the sizes of the microcapsules with 2% of inulin in the present study are similar to those observed by Muthukumarasamy, Allan-Wojtas, and Holley (2006), that obtained an average size for the microcapsules of 2.37 mm with a needle similar to that used in the present study (21G) and with the same $CaCl_2$ (0.5 M) concentration.

Similarly, in the IM method, the size could be also influenced by the stirring speed during the encapsulation process, by the concentration of alginate or other compounds used for the encapsulation, and by the presence of insoluble particles of $CaCO_3$ suspended in the sodium alginate solution that could interfere in the correct emulsion, leading to the production of microspheres with a wide size distribution (Cai et al., 2014). In previous studies with IM, Cai et al. (2014), obtained microcapsule average sizes of 343 μm, that were half size of the capsules in the present work with the same encapsulation method. Song, Yu, Gao, Liu, and Ma (2013), obtained microcapsules with a size between 35 and 373 μm, with an average size of 151.1 μm, similar to the wide range of sizes obtained in the present study for the IM. The size of the capsules and the type of food where they would be used, exert an influence in the sensory perception and thus in the consumer acceptance or non-acceptance of the product. Muthukumarasamy and Holley (2006), used capsules of the same size of the present study (2.1 mm) prepared by extrusion, to be included in sausages. The capsules were visible but were not detected by the panelists as they were similar to the fat particles of the product.

3.2. Bacterial enumeration and survival during storage

The influence of different concentrations of inulin on the survival of *L. plantarum* encapsulated with different methods during storage has not been investigated so far. As it can be observed in Table 1, the viability of *L. plantarum* in both types of microencapsulation remains stable and without significant differences during the first 15 days of storage independently of the presence of inulin. The survival of *L. plantarum* was lower ($\geq$6 log CFU g^{-1}.) in the samples without inulin during the last 15 days of storage regardless of the microencapsulation method ($p < 0.05$). On the other hand, the capsules with 1% of inulin remained without significant differences ($p > 0.05$) respect to the other samples with or without inulin. This could indicate that the amount of inulin incorporated in the capsules is not sufficient as energy source for *L. plantarum* during long storage periods. The samples with 2% of inulin, reached the highest survival values for *L. plantarum* at the end of the 30 days of refrigerated storage at 4 °C (6.66 CFU g^{-1} EMI2% and 6.61 CFU g^{-1} IMI2% ($p < 0.05$). Therefore the 2% of inulin is used as a carbon source by *L. plantarum* after 15 days of storage. Chen, Chen, Liu, Lin, and Chiu (2005) showed that the use of a 1% of peptides and 3% of oligosaccharides using extrusion microencapsulation, improved the protection of the probiotics (*Lactobacillus acidophilus*, *Lactobacillus casei*, *Bifidobacterium longum* and *B. bifidum*) during storage, but the incorporation of isomaltooligosaccharides did not increase the survival of these probiotic microorganisms. In other studies using the spray drying, it was observed that the addition of 10% inulin improved the survival of *Bifidobacterium* BB-12 after microencapsulation (Fritzen-Freire, Prudêncio, Pinto, Muñoz, & Amboni,

Table 1
The effect of method of microencapsulation with different concentration of inulin upon survival of *L. plantarum* (means ± standard deviation)[a] during storage at 4 ± 1 °C.

Days of storage	Number of cells (log CFU g^{-1})					
	EM	EMI1%	EMI2%	IM	IMI1%	IMI2%
0	7.40 ± 0.37^{Ba}	7.38 ± 0.37^{Ba}	7.37 ± 0.37^{Ba}	7.10 ± 0.35^{Ba}	7.34 ± 0.36^{Ba}	7.08 ± 0.35^{Aa}
8	7.21 ± 0.36^{Bab}	7.39 ± 0.37^{Bb}	7.47 ± 0.37^{Bb}	6.58 ± 0.33^{ABa}	7.13 ± 0.36^{Bab}	7.16 ± 0.36^{Aab}
15	6.99 ± 0.35^{Bab}	7.07 ± 0.35^{ABb}	7.40 ± 0.37^{Bb}	6.55 ± 0.32^{ABa}	6.90 ± 0.34^{Bab}	6.97 ± 0.35^{Aab}
30	5.94 ± 0.29^{Aa}	6.34 ± 0.32^{Aab}	6.66 ± 0.33^{Ab}	6.00 ± 0.30^{Aa}	6.19 ± 0.33^{Aab}	6.61 ± 0.38^{Ab}

EM: control extrusion microencapsulation; EMI1%: extrusion microencapsulation with 1% of inulin; EMI2%: extrusion microencapsulation with 2% of inulin; IM: control internal emulsion microencapsulation; IMI1%: internal emulsion microencapsulation with 1% of inulin; IMI2%: internal emulsion microencapsulation with 2% of inulin.
[a–b] Different superscript lower-case letters in the same **row** denote significant differences ($p < 0.05$) among different samples for the same day.
[A–B] Different superscript capital letters in the same **column** denote significant differences ($p < 0.05$) among different days of storage for the same sample.
[a] Mean of triplicates.

Table 2
The effect of method of microencapsulation during storage (4 ± 1 °C) upon survival of *L. plantarum* (means ± standard deviation)[a]

Days of storage	Method of encapsulation	Number of cells (log CFU g^{-1}) Before digestion	After gastric digestion	After intestinal digestion	Number of log Cell reduction (log)
1	EM	8.47 ± 0.42^{Ab}	7.78 ± 0.39^{Aab}	7.56 ± 0.38^{Aa}	0.91
	IM	8.47 ± 0.43^{Ab}	7.54 ± 0.37^{Aa}	7.31 ± 0.36^{Aa}	1.16
15	EM	8.41 ± 0.42^{Ab}	7.80 ± 0.39^{Bab}	7.40 ± 0.37^{Ba}	1.01
	IM	7.83 ± 0.39^{Ab}	6.90 ± 0.34^{Aa}	6.69 ± 0.33^{Aa}	1.14
30	EM	8.25 ± 0.41^{Ab}	7.66 ± 0.38^{Bab}	7.40 ± 0.37^{Ba}	0.85
	IM	7.80 ± 0.39^{Ab}	6.82 ± 0.34^{Aa}	6.53 ± 0.32^{Aa}	1.27

EM: control extrusion microencapsulation; IM: control internal emulsion microencapsulation. [a-b] Different superscript lower-case letters in the same row denote significant differences ($p < 0.05$) for the same method of microencapsulation under simulated gastrointestinal conditions.
[A–B] Different superscript capital letters in the same column denote significant differences ($p < 0.05$) among different method of microencapsulation for the same day of storage under simulated gastrointestinal conditions.
[a] Mean of triplicates.

2013). Therefore, for each microorganism and microencapsulation method, it is very important to consider the type and amount of prebiotic to be used.

3.3. Survival of microencapsulated probiotic in simulated gastric and intestinal juices

The influence of different microencapsulation methods on the protection of probiotic microorganisms during *in vitro* digestion has been studied in previous research (Fritzen-Freire, et al 2013; Krasaekoopt & Watcharapoka, 2014; Muthukumarasamy et al., 2006). However this is the first study on the effect of the time of storage (0, 15 and 30 days) on the survival of microencapsulated *L. plantarum* to the *in vitro* gastrointestinal digestion during 60 min. The survival of *L. plantarum* was measured before and after the simulation of gastric juices and after the intestinal juices respectively (Table 2).

As it is presented in Table 2, the first day it was observed a decrease in the number of cells at the end of the digestion when compared to the initial concentrations of 0.9 and 1.1 logs for EM and IM respectively ($p < 0.05$). Therefore the survival of LP remained without significant differences between both types of microcapsules ($p > 0.05$) during and after the simulated gastrointestinal digestion. However, after 15 and 30 days of storage, the amount of viable cells in EM and IM before the *in vitro* digestion, did not show significant differences ($p > 0.05$). At the end of the gastric digestion, the survival of *L. plantarum* in EM was higher than in IM with values for day 15: 7.80 log CFU g^{-1} EM vs 6.90 log CFU g^{-1} IM and for day 30: 7.66 log CFU g^{-1} EM vs 6.82 log CFU g^{-1} IM. This difference in the survival to the gastric digestion process between the two types of microencapsulation respect to the behavior in the first day of the storage, could be due to the weakening of the structure of the microcapsule during the storage period. After 15 days of storage, the EM capsules resisted better than the IM to the strong conditions of the gastric environment (low pH and pepsin enzymatic activity). Therefore, due to the low resistance of the IM capsules to the conditions of the gastric digestive process, there is a damage of the integrity, leading to the disintegration of the capsules and the eventual release of *L. plantarum*, while the EM capsules remain intact (Fig. 2).

After the intestinal digestion, the survival of *L. plantarum* in IM after 15 and 30 days was lower than in EM. This could be due to the lack of protection of *L. plantarum* by the capsule, as it is free in the medium and subjected to an environmental stress due to the changes in pH and to the interaction with the enzyme pancreatin and bile salts (Table 2). Therefore the survival of *L. plantarum* to the *in vitro* digestion is lower in IM than EM as the time of storage increases (days 15 and 30), with a decrease respect to the viable cells before digestion of 1.01 vs 1.14 logs CFU g^{-1} (day 15) and 0.85 vs 1.27 logs CFU g^{-1} (day 30) respectively. Although the cell concentration is lower in IM than EM, it is above 10^6 CFU g^{-1}after intestinal digestion.

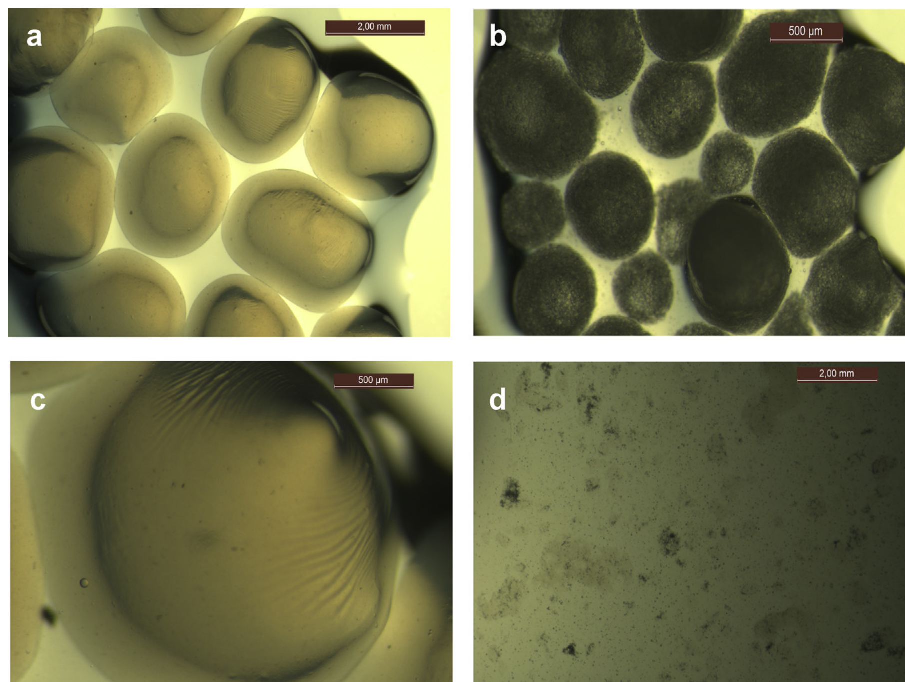

Fig. 2. Stereo microscope images (a) extrusion microcapsules before *in vitro* gastric digestive process (b) internal emulsion microcapsules before *in vitro* gastric digestive process (c) extrusion microcapsules after *in vitro* gastric digestive process (d) internal emulsion microcapsules after *in vitro* gastric digestive process.

Other authors have indicated that the size of the microcapsules has a direct relationship with the survival of the encapsulated microorganism (Muthukumarasamy et al., 2006; Sandoval-Castilla, Lobato-Calleros, García-Galindo, Alvarez-Ramírez, & Vernon-Carter, 2010). According to Sandoval-Castilla et al. (2010), the ability of survival of lactic acid bacteria is positively correlated with the diameter of the capsules using the EM, but without comparing with other encapsulation methods. On the other hand Nualkaekul, Cook, Khutoryanskiy, and Charalampopoulos (2013), after the addition of the capsules to juices refers to the hardness and size of the microcapsules as very important physical characteristics tor either the survival of the microorganism and the sensory characteristics. This author observed a decrease in the extrusion capsules hardness with storage time. However, in the present study the results indicate that the encapsulation method is more relevant respect to the survival of the microorganism to the digestive process, than the size of the microcapsules. Therefore it is necessary the stabilization of the IM capsules to enhance their resistance to the *in vitro* digestion. For this purpose, it would be necessary to reach a balance between the concentrations of alginate, $CaCO_3$ and vegetable oil and to investigate different sizes of microcapsules. Additionally, studies of mechanical resistance during storage of both types of microcapsules would provide some evidence to find the mechanism under the different behaviour of the microcapsules to the gastrointestinal digestion.

4. Conclusions

The size of the microcapsules was affected by the method of encapsulation and by the concentration of inulin. The results during storage showed the improvement of the survival of *L. plantarum* with the use of 2% of inulin for internal emulsion and extrusion capsules, mainly in the two last weeks of refrigerated storage. At the end of the gastrointestinal digestion in both microencapsulation methods, *L. plantarum* had a high survival, above the recommended therapeutic minimum ($\geq 10^6$ CFU g^{-1} or mL^{-1}), though from 15 days of storage, the internal emulsion microencapsulation capsules did not resist to the gastric digestion, losing their integrity and releasing the microorganism to the environment. Thus the extrusion microencapsulation was the best method providing a higher protection and survival for the *L. plantarum* during longer storage periods.

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PUBLICATION 3

Effect of inulin on the viability of *Lactobacillus plantarum* during storage and *in vitro* digestion and on composition parameters of vegetable fermented juices

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ORIGINAL PAPER

Effect of Inulin on the Viability of *L. plantarum* during Storage and *In Vitro* Digestion and on Composition Parameters of Vegetable Fermented Juices

Estefanía Valero-Cases [1] **· María José Frutos** [1]

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Abstract The prebiotic effect of different concentrations of inulin (0, 1 and 2%) on the growth and survival of *Lactobacillus plantarum* (LP) CECT 220 in blended carrot and orange juices was investigated after 24 h of fermentation, during 30 days of storage at 4 °C and through the phases of gastrointestinal digestion after different storage periods. Microbiological and chemical determinations were also carried out in all juices. The lactic fermentation increased the shelf life of the fermented juices with inulin. The hygienic-sanitary quality in fermented juices was better than the control juices. During storage, the inulin improved the viability of LP and the monosaccharide concentration remained higher with respect to the juice without inulin (40% lower). At 30 days, the fermented juices with 2% inulin after *in vitro* digestion presented the highest survival of *L. plantarum*.

Keywords Probiotic · Lactic acid bacteria · Beverages · Chemical parameters · Prebiotic

Introduction

Products containing probiotics and prebiotics are known as synbiotic foods. Probiotics consist mainly of *Lactobacillus* and *Bifidobacterium* strains together with other species such as *Lactococcus*, *Enterococcus* and *Streptococcus* [1, 2]. In addition, these bacteria should be resistant to processing and storage conditions and survive gastrointestinal digestion and be able to reach the colon in sufficient amount [3], hence, their concentration in foods has to be in the range of 10^6–10^7 UFC/mL or gram at the moment of consumption [1]. *Lactobacillus plantarum* is a homo-fermentative lactic acid bacteria (LAB) that produces only lactic acid but can also metabolize a variety of sugars, growing at the same time on different surfaces and substrates such as meat, dairy products and vegetables and frequently in the intestinal tract [4]. Prebiotics are non-digestible food ingredients that are fermented in the colon by beneficial bacteria (*Lactobacillus* and *Bifidobacterium*), stimulating their growth and metabolic activity [5, 6]. Inulin is considered to be a prebiotic and can be added to food without affecting taste to stimulate the LAB [7].

The development of non-dairy probiotic or synbiotic foods from fruits and vegetables has a high potential for the food industry, owing to the growing trend on the market for vegetarian foods, together with the high percentage of lactose intolerant people and the presence of cholesterol in dairy products [8]. Hence, there are nutritional reasons for testing lactic acid fermentation as a potential process for production of fermented juice from fruits and vegetables [9]. During storage of fermented drinks, the low pH, the nutrient depletion and the accumulation of lactic acid is a challenge for the survival of probiotic bacteria being difficult to keep the right microbial doses at the time of consumption [10].

The aim of the present study was to investigate the prebiotic effect of different concentrations of inulin on fermented blended carrot and orange juice, through the measurement of the growth of *L. plantarum* after 24 h of fermentation, during 30 days of refrigerated storage and during simulated human digestion in relation to the storage period. The amounts of inulin, sugars and organic acids were also determined in fermented juices during fermentation and storage.

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Materials and Methods

Bacterial Strain and Culture Conditions

A lyophilized culture of *Lactobacillus plantarum* CECT 220 (LP) was obtained from the Spanish Type Culture Collection (CECT, Valencia, Spain). The bacterial strain was prepared according to the method described by Valero-Cases and Frutos [11]. The lyophilized microorganism was re-suspended in 10 mL of the Man Rogosa Sharpe (MRS) broth (Oxoid; Madrid, Spain) for 48 h at 37 °C (pre-inoculum). To obtain an initial microbial count of around 10^8 colony forming units per mL (CFU/mL), the pre-inoculum (1%, *v*/v) was inoculated in MRS broth and incubated during 24 h at 37 °C. The biomass was harvested by centrifugation at 2000×*g* for 10 min at 4 °C, and washed twice with sterile phosphate buffer saline (PBS) (Oxoid; Madrid, Spain) and stored in 10% (*v*/v) of glycerol at −80 °C until use.

Development and Fermentation of Blended Carrot and Orange Juice Fortified with Inulin

The juice was prepared using 2 kg of carrots (*Daucus carota* L. cv. Nantesa) and 1 kg of oranges (*Citrus sinensis* L. cv. Valencia-Late) from a local market in Orihuela (Alicante). The vegetable material was washed for 5 min with tap water and sodium hypochlorite at 90 °C and immediately rinsed with cold tap water. The carrot juice was obtained using an automatic juice extractor (Vitale Taurus, Taurus Group, Lleida, Spain). The orange juice was extracted with a hand squeezer (Taurus TC8, Taurus Group). The juices were clarified by centrifugation at 5000 × *g* for 5 min at 4 °C. The juice samples were prepared by blending 67% (*v*/v) of carrot juice with 33% (*v*/v) of orange juice, with a final pH of 4.9. Xanthan gum (0.25%, *w*/*v*) (Guinama; Valencia, Spain) was added as a stabilizer.

Artichoke inulin with a degree of polymerization (DP) 10 (Farma-química; Málaga, Spain), was added to the blended juice samples in different proportions to prepare juice without inulin (JIN0%), juice supplemented with 1% inulin (JIN1%) and with 2% inulin (JIN2%). The juices were transferred into sterile borosilicate glass bottles with polypropylene screw caps (250 mL), pasteurized at 90 °C for 5 min in a water bath and cooled to 37 °C in an ice bath. The JIN0%, JIN1% and JIN2% were inoculated with 2.5 mL/250 mL of juice of a suspension of previously prepared LP (10^6 viable cells/mL of juice), and incubated at 37 °C for 24 h. The non-fermented control juice (CJ) was kept in the same conditions during the incubation. After 24 h of incubation, the fermented juices and CJ were stored during 30 days at 4 °C. The samples were analyzed at 0, 1, 8, 10, 12, 15 and 30 days.

Determination of the Viability of *Lactobacillus plantarum*

The viability of LP in the fermented juices was determined by the plate count method. Aliquots (1 mL) of each sample were diluted with 9 mL of sterile peptone water in serial dilutions and they were spread plated in MRS agar (Oxoid; Madrid, Spain) for enumeration and incubated under aerobic conditions at 37 °C for 48 h. The results were expressed as Log_{10} colony forming unit per mL of juice (CFU/mL).

Determination of Yeasts and Moulds

The moulds and yeasts were determined in Petrifilm™ yeast and mould count plates (3 M; Madrid, Spain). The plates were incubated aerobically at 25 °C during 48–72 h for yeasts and 72–140 h for moulds. The results were expressed as Log_{10} CFU/mL.

Survival of *L. plantarum* under Simulated Gastric Juices (SGJ) and Simulated Intestinal Juices (SIJ)

The tolerance of LP to *in vitro* digestion during storage time was determined according to the method described by Valero-Cases and Frutos [11], through the exposition of 10 mL of JIN0%, JIN1% and JIN2% at 37 °C during 60 min to SGJ. These SGJ were prepared in 100 mL of MRS broth acidified to pH 3 with 1 M HCl (Panreac; Barcelona, Spain) and 3 g/L of pepsin (Farma-química; Málaga, Spain). For the preparation of SIJ, the reaction was stopped after 60 min, by adjusting the pH to 7 with 1 M NaOH (Panreac; Barcelona, Spain), 4.5 g/L of bile salts (Sigma-Aldrich; Madrid, Spain) and 1 g/L of pancreatin (Sigma-Aldrich; Madrid, Spain) were added and samples were incubated during 60 min at 37 °C. The survival of LP in each different fermented juice was calculated according to Eq. 1:

$$\text{Survival}\ (\%) = (N_0/N_f) \times 100 \qquad (1)$$

where N_0 is the total number of viable cells in each different fermented juice (JIN0%, JIN1% or JIN2%) before *in vitro* digestion and N_f is the number of viable cells after *in vitro* digestion in each different fermented juice (JIN0%, JIN1% or JIN2%).

Chromatographic Analysis of Sugars, Inulin and Organic Acids

The sugars, inulin and organic acids were simultaneously analyzed using high performance liquid chromatography (HPLC). The determination was made using a Hewlett-Packard HPLC series 1100 instrument (Woldbronn, Germany) equipped with a Supelcogel C-610H (30 cm × 7.8 mm) column and a Supelcoguard C-610H (5 cm × 4.6 mm) guard column

(Supelco, Sigma-Aldrich; Madrid, Spain). A refractive index detector (RID G1362A) was used for the sugars and inulin analysis. The acids were monitored at 210 nm with a visible-ultraviolet (UV-Vis-) diode array detector (DAD G1315A). The mobile phase was orthophosphoric acid at 0.1% and the injection volume was 20 μL with a flow of 0.5 mL/min in isocratic conditions. Identification and quantification were obtained through standard calibration curves for sugars (glucose, fructose, and sucrose), inulin and organic acids (malic, lactic, citric and oxalic acids) (Sigma-Aldrich; Madrid, Spain). The results were expressed as grams per litre.

Statistical Analysis

All experiments and analysis were conducted in triplicate. The results were expressed as mean ± standard deviation. The mean comparison was performed using analysis of variance (ANOVA) followed by a Duncan test ($p < 0.05$), using SPSS v 21.0 software package (SPSS Inc., Chicago, IL, USA).

Results and Discussion

Effect of Inulin on the Growth and Survival of *L. plantarum* and Changes in the Composition of Juices after Fermentation and during Storage

After 24 h of fermentation at 37 °C, the growth of LP was about 9.13 Log_{10} CFU/mL in all juices (Fig. 1a) regardless of the addition of inulin (Fig. 1b, c). At the same time an increase in lactic acid concentration was observed (2 g/L) without significant differences ($p > 0.05$) in samples with inulin and without inulin (Fig. 2). This was associated with a considerable reduction in fructose and glucose, with initial concentrations of 14 g/L decreasing to 9.5 g/L in both monosaccharides at the end of fermentation (Fig. 3). The concentrations of malic acid also decreased from 6.5 to 2 g/L (Fig. 2). This indicates the fast transformation of monosaccharides and the conversion of the malic acid into lactic acid through the malolactic fermentation pathway made by LP after 24 h [12]. Accordingly, the lactic acid production from the metabolism of these substrates resulted in a pH decrease from initial values of 4.9 to 3.9 in all fermented juices after 24 h of fermentation. However, the inulin remained unchanged during this fermentation period (Fig. 1b, c). These results indicate that during fermentation, the main carbon and energy sources for LP were glucose and fructose (Fig. 3), while inulin, with a DP ≥ 10, is fermented more slowly [13, 14]. Thus, the degradation of inulin during fermentation may be related to the food composition, the LAB strain and to the fermentation time. However, between 10 and 15 days of storage, the inulin concentration decreased 17% in JIN1% and JIN2% ($p > 0.05$) (Fig. 1b, c), while the monosaccharides concentrations remained constant during this period for JIN1% and JIN2%. The fermentation of inulin may be favored by the decrease of nutrients after a certain storage period [15]. In the present study, it can be stimulated by the decrease of monosaccharides during the fermentation time and the first eight days of storage. However, in the same storage period, the control samples JIN0% showed a decrease in the concentration of glucose and fructose as the only energy source. After 15 days, the viability of LP was higher for samples with inulin. Nevertheless, the survival of LP in JIN0% started to decrease progressively after 15 days of storage because of the lower concentration of monosaccharides at this stage (Fig. 1a).

During the last 15 days of storage, JIN1% and JIN2% had a lower reduction in the concentration of monosaccharides compared to control samples JIN0% (Fig. 3). This fact could be related to the continuous consumption of inulin by LP during this period, where a decrease in the concentration of inulin was observed in all the samples with inulin (Fig. 1b, c). After 30 days, the total monosaccharide concentration in JIN0% was 4 g/L lower with respect to juices with inulin (Fig. 3). This means that the fermentation of inulin by LP improved the monosaccharide concentration, probably having an impact on the sensory properties. In previous studies, non- fermented blended carrot and orange juices showed high sensory quality in relation to odour and flavour [16, 17]. However, Luckow and Delahunty [18] reported that regular consumers of orange juice detected differences in the odour and flavour of fermented orange juices without prebiotics, being preferred by 11% of consumers. Therefore, more sensory tests must be done during storage in fermented juices with and without inulin in future studies. Nevertheless, in all the fermented juices, the sucrose content did not change after the fermentation period and during the 30 days of refrigerated storage, indicating that sucrose is not metabolized by this strain of LP. However, Kun et al. [19] found that glucose and sucrose were the main carbon and energy sources in carrot juice fermented with *Bifidobacterium* strains. The knowledge on the metabolic use of the different carbon and energy sources is interesting as it depends on the probiotic strain and on the substrate composition.

The citric acid in all the blended carrot and orange juices remained without significant changes ($p > 0.05$) (ca. 3.5 g/L) during fermentation and storage (Fig. 2) meaning that it was not metabolized by LP. Similar results were found in orange juice fermented with LP NCIMB 8826 with the lactic acid being produced after fermentation from the metabolism of sugars, reaching values of 2.7 g/L after six weeks of storage but where the citric acid concentrations remained unchanged [20]. However, other authors reported that the lactic acid in pomegranate juice after 72 h of fermentation ranged between 2 and 6 g/L for different *Lactobacillus* strains (*L. plantarum, L. delbrueckii, L. acidophilus* and *L. paracasei*), the citric acid being the main energy source for them, due to low sugar content in pomegranate juice [21].

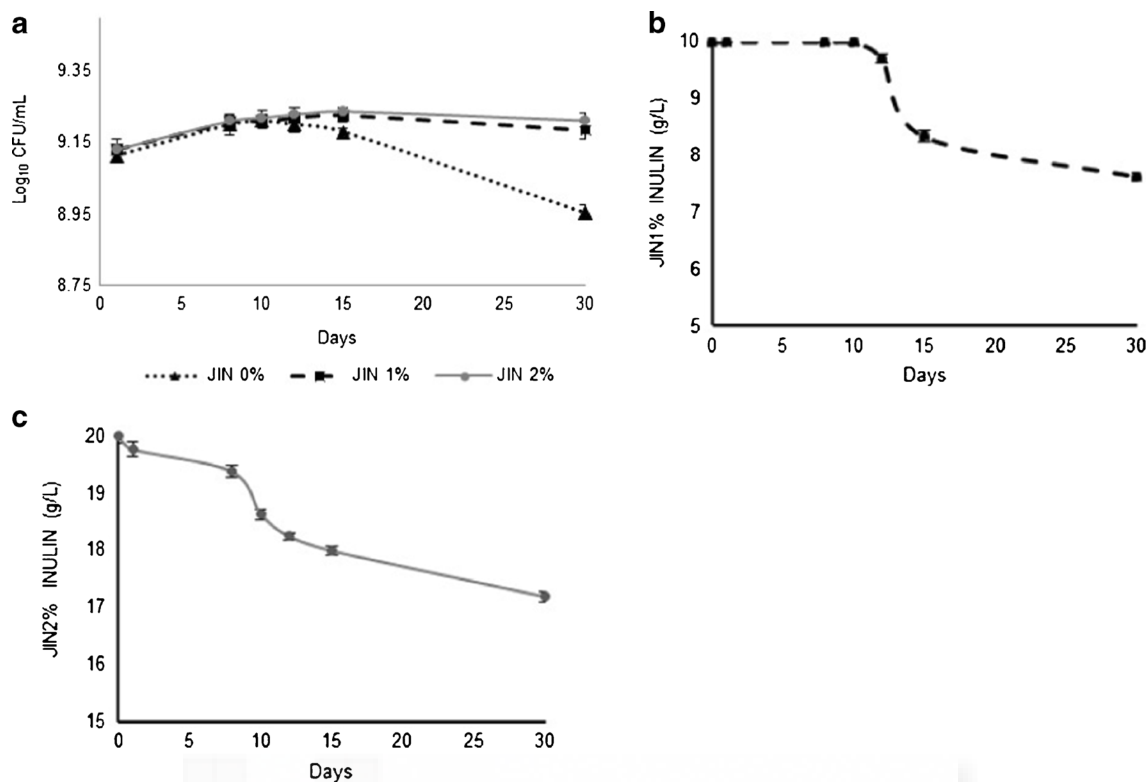

Fig. 1 **a** Effect of different concentrations of inulin on the survival of *L. plantarum* (Log_{10} CFU/mL) in blended carrot and orange juices during refrigerated storage. Evolution of inulin concentrations (g/L) during storage period: **b** Juices with 1% inulin (JIN1%) and **c** Juices with 2% inulin (JIN2%). Data represent average values ± standard deviation of three independent samples, JIN0%, juice without inulin; JIN1%, juice with 1% inulin; JIN2%, juice with 2% inulin

Hence, inulin improved the viability of LP during the last 20 days of refrigerated storage, without showing any significant differences ($p > 0.05$) between JIN1% and JIN2% (Fig. 1b, c). After 30 days of storage, the LP population was 9.2 Log_{10} CFU/mL in JIN1% and JIN2% ($p > 0.05$) and 8.95 Log_{10} CFU/mL in JIN0% (Fig. 1a). Therefore, inulin represents a carbon source available during storage for the LP strain tested and can also protect the microorganism during refrigerated

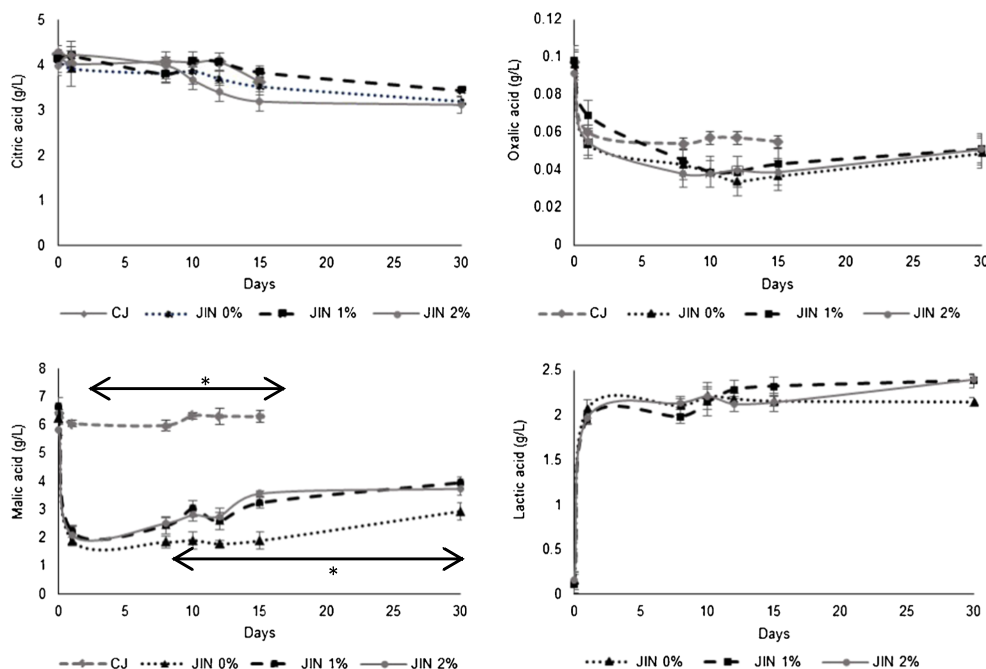

Fig. 2 Changes in individual acid content in blended carrot and orange juices fermented with *L. plantarum* in the presence of different concentrations of inulin, during refrigerated storage, expressed as g/L of juice. Data represent average values ± standard deviation of three independent samples. CJ: control juice; JIN0%: juice without inulin; JIN1%: juice with 1% inulin; JIN2%: juice with 2% inulin. * denotes significant differences between different juices during storage period ($p < 0.05$)

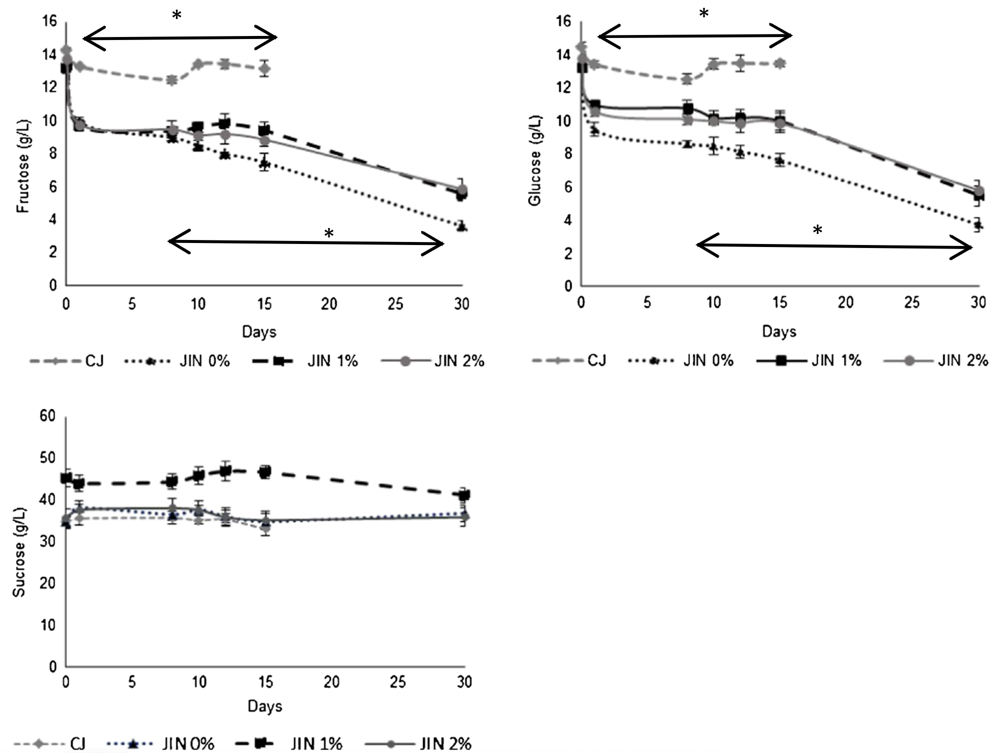

Fig. 3 Changes in sugar content in blended carrot and orange juices fermented with *L. plantarum* in the presence of different concentrations of inulin, during refrigerated storage, expressed as g/L of juice. Data represent average values ± standard deviation of three independent samples. CJ: control juice; JIN0%: juice without inulin; JIN1%: juice with 1% inulin; JIN2%: juice with 2% inulin. * denotes significant differences between different juices during storage period ($p < 0.05$)

storage avoiding cell damage, mainly through physical immobilization of the cells in the inulin structure as this polymer can form aggregates in aqueous media [22, 23]. This protection could be improved by the low precipitation of the semi-dilute particles of inulin during storage, together with the sedimentation of the LP that may increase their interaction leading to a higher protection of the microorganism [24]. Zimeri and Kokini [25], in previous studies reported a 5% of sedimentation of inulin in deionized water after three weeks of storage at room temperature [25]. Valero-Cases and Frutos [11], found similar results after 15 days of storage for the same strain of LP after being microencapsulated with inulin as the only source of energy and reported that 2% of inulin improved the microorganism survival during 30 days of storage compared with 1% of inulin. Paseephol and Sherkat [26] observed that yogurts with inulin improved the viability of *L. casei* but did not have any influence on the survival of *L. delbruecki* subsp. *bulgaricus* and *S. thermophilus*. In milk fermented by different co-cultures of *Lactobacillus* and *Bifidobacterium*, either in pure cultures or in binary co-cultures with *S. thermophilus* or in a cocktail containing all of them an improvement after cold storage in cell viability was observed due to the presence of inulin, depending on the type of microorganism [27], as the ability to ferment inulin is different for every LAB strain [28].

The microbiological analysis of moulds and yeasts showed that they were not detected during the assay in any of the JIN0%, JIN1% and JIN2% juices. Nevertheless, the unfermented juice showed mould and yeast concentrations higher than >3 Log_{10} CFU/mL after 15 days of refrigerated storage. The higher contents of lactic acid in fermented, blended carrot and orange juices contributed to a lower pH and to the increase in shelf life showing a good hygienic-sanitary quality of the samples during the 30 days of refrigerated storage.

Effect of Different Concentrations of Inulin on the Survival of *L. plantarum* in Vegetable Juices under *In Vitro* Digestion during Storage

The LP concentration in fermented juices after 30 days of storage was in the range of the recommended values (10^6–10^7 CFU/mL or gram) [1] for reaching the colon in sufficient concentration after consumption. Therefore, it is interesting to evaluate the effect of inulin on the survival of LP under simulated gastrointestinal digestion at different storage periods (1, 15 and 30 days) and to check the survival through LP concentrations observed during storage (Fig. 4).

Comparing the LP survival in JIN0%, JIN1% and JIN2% under SGJ and SIJ on the first day (Fig. 4), the presence of inulin did not affect the percentage of survival of LP after 120 min of incubation, presenting values of 73% of survival in all fermented juices without significant differences ($p > 0.05$). This represents a decrease of ca. 2.5 logarithmic cycles at the end of the SGJ for this storage period in all samples. However, after *in vitro* digestion at 15 and 30 days of storage, LP showed the same resistance during SGJ, regardless of the presence of inulin. This effect can be due to the preference shown by LAB for simple carbohydrates to resist gastric digestive conditions [29, 30]. Nazzaro et al. [31] found

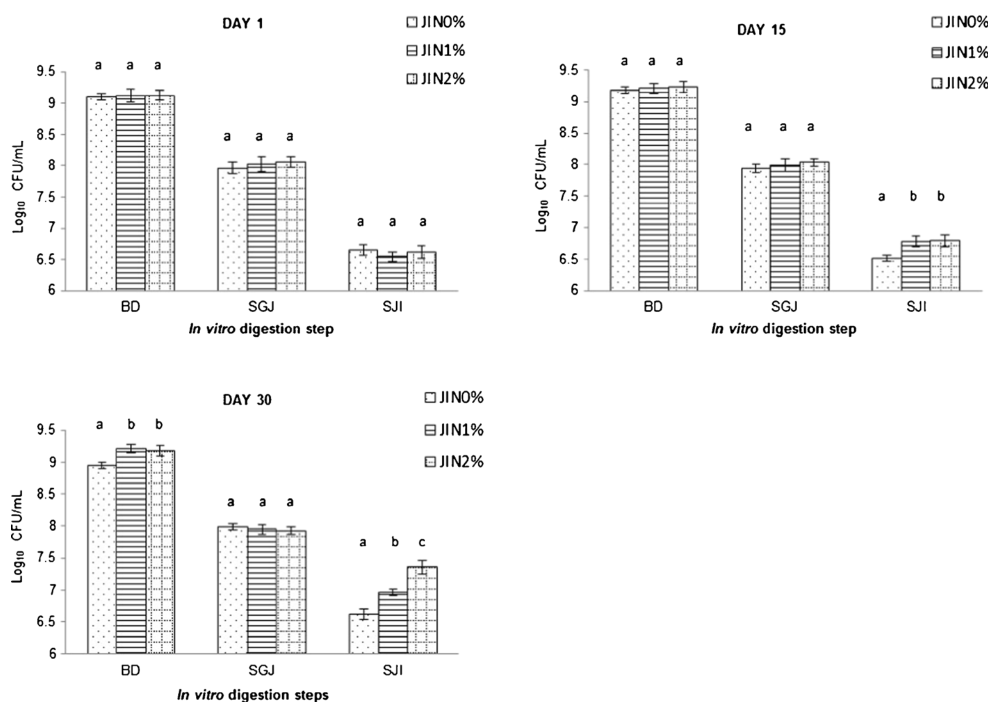

Fig. 4 Effect of different concentrations of inulin on the survival of *L. plantarum* (Log_{10} CFU/mL) in fermented carrot and orange juices during *in vitro* digestion at 1, 15 and 30 days. The error bars represent the standard deviation (n=3). Different letters above the bars denote significant difference on *L. plantarum* survival between three juices for the same step of *in vitro* digestion ($p < 0.05$). JIN0%: juice without inulin; JIN1%: juice with 1% inulin; JIN2%: juice with 2% inulin; BD: Before digestion (0 min); SGJ: after simulated gastric juices (60 min); SJI: after simulated intestinal juice conditions (60 min).

the same results for *L. acidophilus* after SGJ with inulin, pectin and glucose. Nevertheless, during this period, at the end of SIJ, the survival of LP was higher in the juices with inulin. After 15 days, it was observed that the inulin after *in vitro* digestion improved the survival of cells without significant differences ($p > 0.05$) in the concentrations of inulin. However, at 30 days, after *in vitro* digestion it was observed that although the concentration of LP in the JIN0% was high (6.62 Log_{10} CFU/mL), the addition of 1 and 2% of inulin improved the survival of LP, reaching higher values in JIN2% (6.95 and 7.40 Log_{10} CFU/mL for concentrations of 1 and 2% of inulin, respectively). The results demonstrate that the presence of inulin could improve the survival of LP under intestinal conditions for long periods of storage. This could be due to the fact that the period of refrigerated storage time and the intestinal conditions could limit the available sugars [30], favouring the consumption of inulin by LP. The percentage of gastrointestinal survival of LP at the end of the storage was 80% for JIN2%, 75% for JIN1% and 73% for JIN0%. The effect of inulin on the survival of LP during *in vitro* digestion was the same as the one during refrigerated storage as discussed previously. Different food matrices supplemented with inulin have been used in previous studies. In refrigerated synbiotic guava mousses (4 °C) with different amounts of inulin DP 25 (0, 1.33, 2 and 4%) combined with FOS, it has been observed that the samples with inulin improved the survival of *L. acidophilus* after simulated gastrointestinal conditions after 1 and 7 days of storage but not after 14 days [28]. However, in fermented soy products the supplementation with 3% of inulin with a DP 10 did not improve the survival of *L. acidophilus* and *B. animalis* after the gastrointestinal simulation in different storage periods [23]. In the present study, the beneficial effect of inulin on LP survival during the storage and gastrointestinal digestion is mainly observed after long periods of storage. In all cases, there is a high amount of viable cells after the gastrointestinal digestion (> 10^6 CFU/mL).

Conclusion

The present study showed that the hygienic-sanitary quality in fermented juices was better than the control juices for long storage periods. The fermented, blended carrot and orange juice could be a good matrix for the delivery of *L. plantarum* at high concentrations (>10^6 CFU/mL) in the colon. However, during long storage periods, the fermented juices with 2% of inulin showed the best survival of *L. plantarum* after *in vitro* digestion. During storage, the inulin leads to the highest *L. plantarum* survival (regardless of the concentration) and to the highest monosaccharide content (40% higher).

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no competing interests.

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Development of prebiotic nectars and juices as potential substrates for *Lactobacillus acidophillus*: Special reference to physicochemical characterization and consumer acceptability during storage

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Development of prebiotic nectars and juices as potential substrates for *Lactobacillus acidophilus:* Special reference to physicochemical characterization and consumer acceptability during storage

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The aim of the present study was to investigate the effects of physicochemical parameters and inulin supplementation on the viability of *Lactobacillus acidophilus* and on the sensory acceptance of the product. Carrot-orange juices and nectars were fermented for 36 h then stored for 40 days at 4 °C. The results showed that the growth of *L. acidophilus* was strongly influenced by the beverage composition. The lower monosaccharides concentration in nectars led to a better consumption of inulin by the microorganism because it was the main source of energy during fermentation. However, inulin was not fermented in juices during this period and the only substrates used by *L. acidophilus* were the monosaccharides. During storage, inulin was used as a prebiotic in all beverages, but it only improved the *L. acidophilus* survival in nectars. The extent of lactic acid production depended on the composition (sugars, inulin and malic acid). All the beverages were a good medium to keep the *L. acidophilus* survival above the minimum recommended (10^6–10^7 CFU/mL) during the storage. The fermented nectars were preferred by consumers respect to the other beverages due to the effect of the inulin, sucrose and water addition on sweetness and acidity during storage.

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1. Introduction

Many products in the food market in Europe, US and Japan are functional foods, with functional dairy products being the most common (Marsh, Hill, Ross, & Cotter, 2014). However, efforts are currently being made to offer consumers an alternative to fermented milk products. Fruit and vegetable drinks are a good choice for all ages, because they are an excellent source of antioxidant vitamins, bioactive compounds and minerals, and furthermore, they are refreshing and have attractive flavours (Simsek, El, Kancabas Kilinc, & Karakaya, 2014; Vijaya Kumar, Vijayendra, & Reddy, 2015). These drinks, unlike dairy products, do not have milk allergens, which cannot be consumed by certain sectors of the population (Luckow and Delahunty 2004). Probiotics are live microorganisms (mainly strains of *Lactobacillus* and *Bifidobacterium*) which when administered in adequate amounts (10^6–10^7 CFU/mL or g), confer health benefits to the host (FAO/WHO, 2002). *Lactobacillus acidophilus*, is the prevalent *Lactobacillus* in the intestinal tract of healthy humans, and it is associated with intestinal microbial balance (Arihara et al., 1998).

A prebiotic is a "non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, thereby improving host health" (Gibson & Roberfroid, 1995). Inulin is a plant reserve polysaccharide, which the human digestive system cannot breakdown due to the β (2 → 1) links between the fructose molecules (Li, Li, Wang, Du, & Qin, 2012).

When developing fermented fruit and vegetable beverages the substrates and the type and concentration of carbohydrates are important parameters that need to be carefully controlled (Marsh et al., 2014). At the same time, the lactic acid fermentation of fruits and vegetables matrices is an interesting strategy for improving the nutritional and sensory characteristics of beverages protecting them from the microbiological spoilage (Di Cagno, Coda, De Angelis, & Gobbetti, 2013; Pimentel, Madrona, Garcia, & Prudencio, 2015a).

To the best of our knowledge, this is the first comparative study between fermented prebiotic juices and fermented prebiotic

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beverages made with addition of water and sugar to juice (nectars). Therefore, the aim of the present study was to investigate the effects of physicochemical parameters and inulin supplementation on the viability of *Lactobacillus acidophilus* CECT 4356 and on the sensory acceptance of the product. Carrot-orange juices and nectars were fermented with *L. acidophilus* for 36 h then stored for 40 days at 4 °C.

2. Material and methods

2.1. Culture preparation

L. acidophilus CECT 903 (ATCC4356) was purchased from the Spanish Type Culture Collection (CECT, Valencia, Spain). The lyophilized microorganism was re-suspended in 10 mL of the Man Rogosa Sharpe (MRS) broth (Oxoid; Madrid, Spain) for 24 h at 37 °C (pre-inoculum) according to Valero-Cases and Frutos (2015) with some modifications: The pre-inoculum (1% v/v) was inoculated in MRS broth and incubated during 24 h at 37 °C to obtain an initial microbial count of around 8 Log Colony Forming Units per mL (CFU/mL). The biomass was separated by centrifugation at 2000×*g* for 10 min at 4 °C, and washed twice with sterile phosphate buffer saline (PBS) (Oxoid; Madrid, Spain) to remove the residual MRS broth. The L. *acidophilus* was re-suspended in PBS at a cell concentration of approximately 9 Log CFU/mL and stored in glycerol (10% v/v) at −80 °C until use. These stocks were used as inoculums of activated probiotic culture (*L. acidophilus* CECT 4356).

2.2. Preparation and fermentation of carrot-orange juices and nectars supplemented with different concentrations of inulin

Carrots (*Daucus carota* L.) cv. Nantesa and oranges (*Citrus sinensis* L.) cv. Valencia-Late were washed for 5 min with tap water at 90 °C with sodium hypochlorite (2 mL/L of water) and immediately rinsed in an ice bath. The carrots were processed with an automatic juice extractor (Vitale Taurus, Spain) and the oranges with a hand squeezer (Taurus TC8, Spain). The juices obtained were clarified by centrifugation at 5000×*g* for 5 min at 4 °C.

The carrot-orange juices were prepared by blending 50% (v/v) of carrot and 50% (v/v) orange juices and were used as control juices (CJ). The prebiotic juices were supplemented with different proportions of artichoke inulin with a degree of polymerization (DP) 10 (Farma-química; Málaga, Spain), to get the following fermented juices: juice without inulin (JI0%), juice supplemented with 1% inulin (JI1%) and with 3% inulin (JI3%).

The carrot-orange control nectar (CN) was prepared with 50% (v/v) of control orange–carrot juice, 42.5% (v/v) of mineral water and 7.5% (w/v) of food grade sucrose. To obtain the prebiotic nectars, the inulin was added in different proportions: nectar without inulin (NI0%), nectar supplemented with 1% inulin (NI1%) and nectar supplemented with 3% inulin (NI3%).

Citric acid was the major acid in both beverage types prior to fermentation, therefore, to ensure the same initial pH, it was used to adjust the pH to 3.5 in all beverages.

All the samples were put in sterile borosilicate glass bottles (100 mL) with polypropylene screw cap, sterilized in a water bath at 100 °C for 15 min and cooled to 37 °C in an ice bath.

2.3. Preparation of fermented juices and nectars

Each sample (JI0%, JI1%, JI3%, NI0%, NI1%, NI3%) was inoculated with 1% (v/v) of *Lactobacillus acidophilus* suspension previously prepared, corresponding to 6 Log CFU of viable cells/mL of beverage. The fermentation was performed at 37 °C for 36 h. The non-fermented control juice (CJ) and non-fermented control nectar (CN) were kept in the same conditions during the incubation time (37 °C for 36 h). After fermentation, all drinks were stored for 40 days at 4 °C (to stop the fermentation and improve the shelf life of the beverages). Samples were taken at 0, 10, 20, 30 and 40 days for analyses.

2.4. Growth and survival of L. acidophilus during refrigerated storage

L. acidophilus viability was determined after fermentation and during 40 days of storage in the different fermented juices and nectars. Aliquots of each fermented beverage were removed and 10-fold serial dilutions in sterile peptone water were spread in triplicate on MRS agar plates in anaerobic conditions (AnaeroJar™ 2.5 L, Oxoid, Spain) and incubated in anaerobic bags (AnaeroGen™ 2.5 L, Oxoid, Spain) at 37 °C for 48 h. The results were expressed as Log CFU/mL of beverage.

2.5. Quantification of moulds and yeasts

The concentrations of yeasts and moulds were determined using Petrifilm™ yeasts and moulds count plates (3 M; Madrid, Spain). The plates were incubated aerobically at 25 °C for 48–72 h to determine the yeasts concentration and for 72–140 h to determine the moulds concentration. The results were expressed as Log CFU/mL.

2.6. Preparation of the samples

Each type of juice and nectar was diluted with ultrapure water 1:10 (v/v) and the mixtures were homogenized using an Ultra-Turrax (IKA, T25D, Staufen, Germany) at 15000×*g* for 5 min. The samples were centrifuged at 10000×*g* for 10 min at 4 °C and the supernatants were filtered (0.45 µm, Millipore, Spain) into glass vials and stored at −20 °C until analysis.

2.7. Chromatographic identification and quantification of sugars, inulin and organic acids

Sugars, inulin and organic acids were simultaneously analyzed in all nectars and juices by high performance liquid chromatography (HPLC) using a Hewlett-Packard HPLC series 1100 instrument (Woldbronn, Germany) equipped with a Supelcogel C-610H (30 cm × 7.8 mm) column and a Supelcoguard C-610H (5 cm × 4.6 mm) guard column (Supelco, Sigma; Madrid, Spain). The acids were measured using a visible-ultraviolet (UV–vis) diode array detector (DAD G1315A) at 210 nm. For the sugars and inulin analysis, a refractive index detector (RID G1362A) was used. The mobile phase was 0.1% orthophosphoric acid, the injection volume was 20 µL, and the flow rate was 0.5 mL/min in isocratic conditions. The concentrations were calculated using calibration curves with standards for sugars (glucose, fructose, and sucrose), organic acids (malic, lactic, citric, acetic and oxalic acids) and inulin (Sigma; Madrid, Spain). The results were expressed as grams per litre (g/L).

2.8. Physicochemical analysis

The soluble solids content (TSS) was determined using a digital refractometer (Hanna®, model HI96812) with a scale of 0–75% and expressed as °Brix. The pH and titratable acidity (TA) were determined using an automatic titrator (TritoMatic®, Crison) calibrated with buffers at pH 4.00 and 7.00 at 25 °C. Analysis was done by titration with 1 g of each sample in 9 mL ultrapure water with 0.1 N NaOH solution and expressed as percentage (%) of lactic acid for fermented samples and as percentage (%) of citric acid for control

samples.

2.9. Sensory evaluation

Sensory evaluation of fermented nectars and juices was made before storage and after 20 and 40 days at 4 °C. The sensory acceptance study was performed by 40 untrained panelists (18 women and 22 men aged between 18 and 55 years old) (Salmerón, Thomas, & Pandiella, 2015). The consumers were recruited among students and staff of the High Polytechnic School of the Miguel Hernández University (Orihuela, Alicante, Spain), all of them consumers of fruit juices. During sessions, 30 mL of each sample were presented in a random order to the panelists in transparent plastic glasses (with a three digit code) at 4 °C. An unstructured 9-point hedonic scale (1 = dislike very much to 9 = like very much) was used in order to determine the acceptance of different attributes (colour, flavour, aroma, acidity and sweetness) and the overall acceptance. The consumers were not informed about the beverage fermentation.

2.10. Statistical analysis

All experiments and analyses were made in triplicate. The results were expressed as mean ± standard deviation. The mean comparison was performed using analysis of variance (ANOVA) followed by a Duncan multiple range test to evaluate the significant differences ($p < 0.05$), using SPSS v 21.0 software package (SPSS Inc., Chicago-Illinois-USA).

3. Results and discussion

3.1. Metabolism of sugars and inulin and viability of L. acidophilus after fermentation and during storage

Table 1 shows the sugars content in different beverages before and after fermentation and during the 40 days of refrigerated storage. Before fermentation, sucrose was the sugar present in highest concentration in all beverages (Table 1). The sucrose contents in nectars (around 80 g/L) were twice the amount in juices (around 40 g/L), while glucose and fructose concentrations in all juices (19–21 g/L) were higher than in nectars (13–14 g/L). At the same time, TSS in control nectars were higher than in control juices, with values of 12 and 10 °Brix, respectively (Table 2). The sugars concentrations and TSS were different between control nectars and juices, due to the presence of sucrose and water in the nectar formulations. The TSS in nectars were similar to the results reported by other authors for apple beverages supplemented with sugar or prebiotics (de Souza Neves Ellendersen, Granato, Bigetti Guergoletto, & Wosiacki, 2012; Pimentel, Madrona, Garcia, et al., 2015a). Inulin supplementation contributed to an increase in TSS in all beverages, probably due to the presence of monosaccharides such as D-fructose in the inulin composition. Pimentel, Madrona, and Prudencio (2015a), also observed an increase in TSS when oligofructose was added to clarified apple juice because of the presence of mono and disaccharides in the oligofructose composition.

After 36 h of fermentation at 37 °C, the juices without inulin were a better substrate for *L. acidophilus* growth than the nectars without inulin (NI0%), the concentrations of viable cells after fermentation were 8.34 and 8.03 Log CFU/mL in juices and nectars, respectively (Table 3). Therefore, the juices and nectars were good matrices to reach a high concentration of *L. acidophilus* without any further supplementation. However, physicochemical stability assessment was needed to confirm that the fermented and non-fermented beverages remained with similar characteristics during storage (Pimentel, Madrona, Garcia, et al., 2015a), maintaining at the same time suitable concentrations of *L. acidophilus*. Hence, inulin was used in different concentrations with the aim of improving the chemical parameters of stability. The results showed that the *L. acidophilus* growth was boosted in some of the beverages depending on the composition. When comparing the effect of inulin addition on *L. acidophilus* viability among the nectars, it was observed that *L. acidophilus* reached the maximum viability (8.30 Log CFU/mL) in the nectars with the highest inulin supplementation (3%), at the same time, these samples showed a decrease of 6 g/L of inulin (Fig. 1). NI1% had a *L. acidophilus* viability of 8.25 Log CFU/

Table 1
Sugar content in different formulations of juices and nectars.[a]

Sugars	Storage time (days)	Formulations[b]							
		CJ	JI0%	JIN1%	JIN3%	CN	NI0%	NI1%	NI3%
Sucrose (g/L)	0	38.66 ± 0.13Aa	38.52 ± 0.13Ac	38.71 ± 0.11Ab	38.71 ± 0.11Aa	80.18 ± 0.24Ba	80.29 ± 0.27Bd	80.28 ± 0.22Bd	80.22 ± 0.81Bc
	1 (AI)	38.69 ± 0.01Aa	38.44 ± 0.38Ac	38.66 ± 0.20Ab	38.44 ± 0.38Aa	80.18 ± 0.24Ca	77.93 ± 0.53Bc	80.28 ± 0.18Cd	80.17 ± 0.08Cc
	10	38.62 ± 0.08Ba	37.37 ± 0.13Ab	38.62 ± 0.13Bb	38.48 ± 0.38Ba	80.16 ± 0.28Fa	73.24 ± 0.35Cb	76.67 ± 0.02Dc	78.34 ± 0.77Eb
	20	38.62 ± 0.08Ba	37.16 ± 0.01Ab	38.40 ± 0.28Bb	38.45 ± 0.23Ba	80.16 ± 0.28Fa	73.05 ± 0.14Cb	76.08 ± 0.26Dbc	77.75 ± 0.30Eb
	30	38.67 ± 0.06Ca	35.99 ± 0.17Aa	37.80 ± 0.12Ba	38.49 ± 0.25Ca	80.18 ± 0.24Ga	72.98 ± 0.17Dab	75.67 ± 0.47Eab	77.63 ± 0.04Fb
	40	38.64 ± 0.08Ca	35.56 ± 0.03Aa	37.40 ± 0.03Ba	38.45 ± 0.25Ca	80.18 ± 0.23Ga	72.31 ± 0.23Da	75.34 ± 0.65Ea	73.52 ± 0.09Fa
Glucose (g/L)	0	19.15 ± 0.87Ba	19.16 ± 0.88Bf	19.16 ± 0.88Bd	19.16 ± 0.88Bd	13.48 ± 0.22Aa	13.48 ± 0.09Ac	13.48 ± 0.18Ac	13.47 ± 0.23Ad
	1 (AI)	19.09 ± 0.10Ea	16.31 ± 0.04De	16.24 ± 0.34Dc	15.47 ± 0.17Cc	13.48 ± 0.21Ba	13.09 ± 0.04Ab	13.34 ± 0.05ABbc	13.14 ± 0.01ABc
	10	19.09 ± 0.00Ga	16.29 ± 0.34Fd	15.73 ± 0.16Eb	15.46 ± 0.20Dc	13.54 ± 0.14Ca	12.95 ± 0.08Ab	13.12 ± 0.08Bb	12.90 ± 0.00Abc
	20	19.09 ± 0.02Fa	15.72 ± 0.24Eb	15.71 ± 0.67Eb	15.43 ± 0.35Dc	13.54 ± 0.14Ca	12.18 ± 0.02Aa	12.55 ± 0.12Ba	12.80 ± 0.02Bb
	30	19.10 ± 0.01Fa	15.47 ± 0.21Ea	15.61 ± 0.21Eab	15.18 ± 0.01Db	13.48 ± 0.13Ca	12.02 ± 0.00Aa	12.65 ± 0.02Ba	12.47 ± 0.01Ba
	40	19.09 ± 0.18Ha	16.05 ± 0.01Gc	15.28 ± 0.15Fa	14.91 ± 0.14Ea	13.50 ± 0.18Da	12.00 ± 0.00Aa	12.62 ± 0.12Ca	12.37 ± 0.06Ba
Fructose (g/L)	0	20.85 ± 0.12Ba	20.86 ± 0.13Bf	20.86 ± 0.13Bd	20.86 ± 0.13Bd	13.78 ± 0.58Aa	13.74 ± 0.00Ae	13.75 ± 0.07Ad	13.77 ± 0.00Af
	1 (AI)	21.04 ± 0.47Da	18.18 ± 0.03Ce	18.42 ± 0.25Cc	18.52 ± 0.82Cc	13.63 ± 0.27Ba	12.34 ± 0.03Ad	13.54 ± 0.08Bc	13.63 ± 0.02Be
	10	21.06 ± 0.81Ha	17.52 ± 0.01Ec	17.82 ± 0.20Fb	17.93 ± 0.00Gb	13.80 ± 0.01Da	12.34 ± 0.02Ad	12.99 ± 0.01Bb	13.47 ± 0.11Cd
	20	20.77 ± 0.04Ha	17.01 ± 0.38Eb	17.34 ± 0.47Fa	17.68 ± 0.95Gb	13.80 ± 0.01Da	11.91 ± 0.05Ac	12.43 ± 0.10Ba	13.16 ± 0.05Cc
	30	20.74 ± 0.52Ha	16.84 ± 0.10Ea	17.30 ± 0.46Fa	17.55 ± 0.33Gb	13.78 ± 0.00Da	11.37 ± 0.03Ab	12.41 ± 0.04Ba	12.79 ± 0.03Cb
	40	20.72 ± 0.30Fa	17.70 ± 0.03Ed	17.28 ± 0.39Ea	16.95 ± 0.46Da	13.78 ± 0.01Ca	11.28 ± 0.02Aa	12.41 ± 0.15Ba	12.35 ± 0.03Ba

AI, after incubation period (36 h at 37 °C).

[a] Means ± standard deviation in the same column followed by different lowercase letters indicate statistically significant differences at ($p \leq 0.05$) for each formulation affected by the storage time (n = 3). Means ± standard deviation in the same row followed by different uppercase letters indicate statistically significant differences at ($p \leq 0.05$) between formulations for the same storage time (n = 3).

[b] Formulations: CJ; unfermented control juices; JIN0%, juices without inulin; JIN1%, juices + 1% of inulin; JIN3%, juices + 3% of inulin; CN, unfermented control nectars; N0%, nectar without inulin; NI1%, nectar + 1% of inulin; NI3%, nectar + 3% of inulin.

Table 2
Physicochemical parameters (pH, titratable acidity and TSS) in different formulations of juices and nectars.[b]

Parameters	Storage time (days)	Formulations[c]							
		CJ	JI0%	JIN1%	JIN3%	CN	NI0%	NI1%	NI3%
pH	0	3.50 ± 0.02Aa	3.50 ± 0.01Ad	3.50 ± 0.01Ad	3.50 ± 0.01Ae	3.50 ± 0.02Aa	3.50 ± 0.02Ae	3.50 ± 0.01Af	3.50 ± 0.01Ad
	1 (AI)	3.50 ± 0.01Ba	3.26 ± 0.07Ac	3.34 ± 0.13Ac	3.23 ± 0.06Ad	3.50 ± 0.01Ba	3.26 ± 0.01Ad	3.20 ± 0.01Ae	3.25 ± 0.01Ac
	10	3.50 ± 0.01Ba	3.09 ± 0.01Ab	3.07 ± 0.01Ab	3.07 ± 0.01Ac	3.50 ± 0.01Ba	3.07 ± 0.03Ac	3.08 ± 0.02Ad	3.09 ± 0.02Ab
	20	3.50 ± 0.02Ea	2.99 ± 0.01B[a]	3.00 ± 0.01Bab	3.00 ± 0.01Bb	3.50 ± 0.01Ea	2.97 ± 0.02Ab	3.03 ± 0.02Cc	3.05 ± 0.03Db
	30	3.50 ± 0.01Da	3.04 ± 0.01Cab	2.99 ± 0.01Bab	2.97 ± 0.01Bb	3.50 ± 0.01Da	2.89 ± 0.03Aa	2.92 ± 0.01Ab	2.90 ± 0.01Aa
	40	3.50 ± 0.02Ea	2.97 ± 0.05Da	2.93 ± 0.04CDa	2.89 ± 0.01BCa	3.50 ± 0.05Ea	2.89 ± 0.03BCa	2.82 ± 0.01Aa	2.87 ± 0.05ABa
Titratable acidity [a]	0	0.59 ± 0.01Aa	0.59 ± 0.01Aa	0.59 ± 0.01Aa	0.59 ± 0.01Aa	0.59 ± 0.01Aa	0.59 ± 0.01Aa	0.59 ± 0.01Aa	0.59 ± 0.01Aa
	1 (AI)	0.59 ± 0.01Aa	1.38 ± 0.11Cb	1.29 ± 0.03Cb	1.32 ± 0.06Cb	0.59 ± 0.05Aa	1.09 ± 0.02Bb	1.04 ± 0.04Bb	1.06 ± 0.04Bb
	10	0.59 ± 0.01Aa	1.41 ± 0.05Cb	1.42 ± 0.03Cc	1.36 ± 0.02Cbc	0.59 ± 0.01Aa	1.18 ± 0.01Bc	1.22 ± 0.01Bc	1.22 ± 0.06Bc
	20	0.59 ± 0.02Aa	1.46 ± 0.04Db	1.42 ± 0.02Dc	1.39 ± 0.06Cbc	0.59 ± 0.0A1a	1.26 ± 0.0B3d	1.25 ± 0.04Bc	1.23 ± 0.02Bc
	30	0.59 ± 0.01Aa	1.44 ± 0.01Db	1.42 ± 0.02Dc	1.45 ± 0.01Dc	0.59 ± 0.01Aa	1.31 ± 0.01Ce	1.32 ± 0.04Cc	1.25 ± 0.03Bc
	40	0.59 ± 0.01Aa	1.46 ± 0.37Eb	1.41 ± 0.01Dc	1.43 ± 0.07DEc	0.59 ± 0.01Aa	1.31 ± 0.01Ce	1.26 ± 0.01BCd	1.25 ± 0.02Bc
Total Soluble Solids (°Brix)	0	10.00 ± 0.03Aa	10.03 ± 0.05Ac	11.03 ± 0.05Bb	12.00 ± 0.05Ca	12.00 ± 0.05Ca	12.03 ± 0.05Cc	13.00 ± 0.05Dc	14.00 ± 0.05Ec
	1 (AI)	10.00 ± 0.05Aa	10.03 ± 0.05Ac	11.03 ± 0.05Bb	12.00 ± 0.05Da	12.00 ± 0.05Da	11.63 ± 0.05Cb	13.00 ± 0.05Ec	14.00 ± 0.05Fc
	10	10.00 ± 0.04Ba	9.70 ± 0.05Ab	11.03 ± 0.05Db	12.00 ± 0.05Ea	12.00 ± 0.05Ea	10.30 ± 0.05Ca	12.50 ± 0.05Fb	13.70 ± 0.05Gb
	20	10.00 ± 0.05Ba	9.67 ± 0.05Ab	11.03 ± 0.05Db	12.00 ± 0.05Ea	12.00 ± 0.05Ea	10.87 ± 0.05Ca	12.40 ± 0.05Fab	13.60 ± 0.05Gb
	30	10.00 ± 0.05Ba	9.43 ± 0.05Aa	10.67 ± 0.05Ca	12.00 ± 0.05Ea	12.00 ± 0.05Ea	10.87 ± 0.05Da	12.30 ± 0.05Fa	13.60 ± 0.05Gb
	40	10.00 ± 0.05Ba	9.43 ± 0.05Aa	10.63 ± 0.05Ca	12.00 ± 0.05Ea	12.00 ± 0.05Ea	10.80 ± 0.05Da	12.30 ± 0.05Fa	13.00 ± 0.05Ga

AI, after incubation period (36 h at 37 °C).

[a] Titratable acidity was expressed as % of lactic acid for fermented samples after 1(AF) and as % of citric acid for control samples.

[b] Means ± standard deviation in the same column followed by different lowercase letters indicate statistically significant differences at ($p \leq 0.05$) for each formulation affected by the storage time (n = 3). Means ± standard deviation in the same row followed by different uppercase letters indicate statistically significant differences at ($p \leq 0.05$) between formulations for the same storage time (n = 3).

[c] Formulations: CJ; unfermented control juices; JIN0%, juices without inulin; JIN1%, juices +1% of inulin; JIN3%, juices +3% of inulin; CN, control nectars; N0%, nectar without inulin; NI1%, nectar +1% of inulin; NI3%, nectar +3% of inulin.

Table 3
Viability (Log CFU/mL) of *Lactobacillus acidophilus* in different formulations of juices and nectars.[a]

Storage time (days)	Viable cells (Log CFU/mL) in different formulations[b]					
	JI0%	JI1%	JI3%	N0%	NI1%	NI3%
1 (AI)	8.34 ± 0.11Dd	8.35 ± 0.16Cd	8.35 ± 0.10Dd	8.03 ± 0.40Aa	8.25 ± 0.07Db	8.30 ± 0.12Ec
10	8.01 ± 0.03Ca	7.97 ± 0.16Ba	8.05 ± 0.05Ca	7.97 ± 0.66Aa	7.96 ± 0.66Ca	7.99 ± 0.32Da
20	7.75 ± 0.44Abc	7.77 ± 0.05Cc	7.79 ± 0.00Bc	7.61 ± 0.23Ba	7.70 ± 0.00Bb	7.76 ± 0.27Cc
30	7.81 ± 0.19Bc	7.77 ± 0.05Cc	7.82 ± 0.33Bc	7.61 ± 0.30Ba	7.68 ± 0.19ABb	7.69 ± 0.19Bb
40	7.77 ± 0.05ABb	7.74 ± 0.39Cb	7.72 ± 0.35Ab	7.62 ± 0.52Ba	7.63 ± 0.22Aa	7.64 ± 0.14Aa

AI, after incubation period (36 h at 37 °C).
[a] Means ± standard deviation in the same column followed by different uppercase letters indicate statistically significant differences at ($p \leq 0.05$) for each formulation affected by the storage time (n = 3). Means ± standard deviation in the same row followed by different lowercase letters indicate statistically significant differences at ($p \leq 0.05$) between formulations for the same storage time (n = 3).
[b] Formulations: JIN0%, juices without inulin; JIN1%, juices + 1% of inulin; JIN3%, juices + 3% of inulin; N0%, nectar without inulin; NI1%, nectar + 1% of inulin; NI3%, nectar + 3% of inulin. Values followed by the different letter within the same column or row were statistically different ($p \leq 0.05$).

mL and a decrease of 2 g/L of inulin. However, inulin supplementation in juices did not influence the *L. acidophilus* concentration after fermentation and it was still significantly higher ($p < 0.05$) than in the nectars. The decrease in fructose and glucose concentrations in all the fermented nectars was lower than in fermented juices, with values in the range of 1–2% (w/v) and 3–4% (w/v) respectively (Table 1). Nevertheless, the lower initial concentration of monosaccharides in nectars led to differences in the sucrose and inulin fermentations by *L. acidophilus* with respect to the juices. In nectars without inulin, the *L. acidophilus* fermented the sucrose, with a higher decrease (3%) with respect to the synbiotic nectars resulting in a significant decrease in TSS level. In contrast, in prebiotic nectars inulin was the preferred substrate for *L. acidophilus*

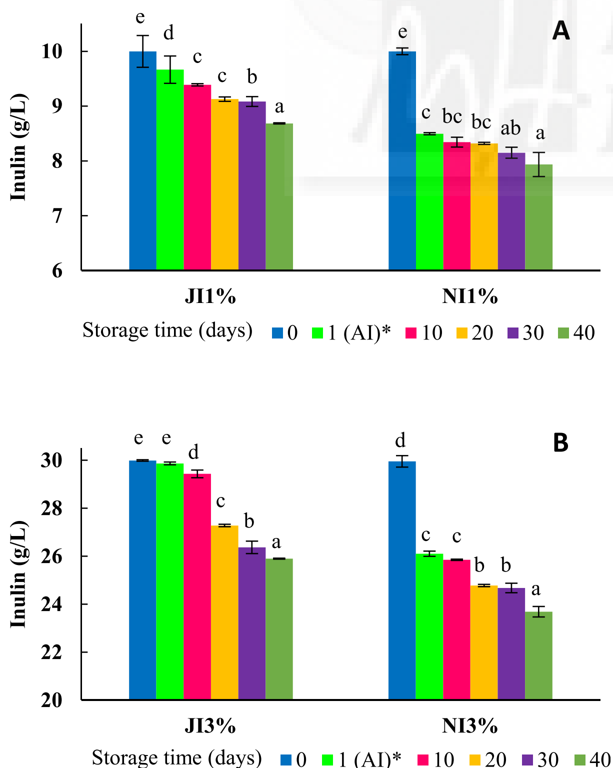

Fig. 1. Inulin concentration (g/L) during storage in fermented juices and nectars supplemented with 1% of inulin (A): juices with 1% of inulin (JIN1%); nectar with 1% of inulin (NI1%) and in fermented juices and nectars supplemented with 3% of inulin (B): juices with 3% of inulin (JIN3%); nectar with 3% of inulin (NI3%). The error bars represent the standard deviation (n = 3). Different lowercase letters indicate statistically significant differences at ($p \leq 0.05$) for each formulation affected by the storage time (days). *AI, after incubation period (36 h at 37 °C).

metabolism and was the main source of energy during fermentation. However, inulin was not fermented in juices, and monosaccharides were the only substrates metabolized by *L. acidophilus* during this period. In previous studies with fermented dairy drinks, it has been reported that in absence of sucrose, *L. acidophilus* can use other sources of energy such as glucose and lactose for lactic fermentation (Gomes de OliveiraI, Fernandes GarciaII, de Cássia Ramos do Egypto QueirogaI, & Leite de SouzaII, 2012).

After 10 days of refrigerated storage no significant difference in the survival of *L. acidophilus* was observed between the fermented samples (Table 3), and a high concentration of 7.9 Log UFC/mL ($p > 0.05$) was maintained in all samples during this period. The inulin concentration did not change in any of the beverages during storage because the *L. acidophilus* metabolized monosaccharides and sucrose as the main energy sources in juices and nectars. However, after 20 days of storage, the highest survival of *L. acidophilus* (7.79 Log CFU/mL) was observed in the juices with 3% of inulin together with the highest inulin consumption (around 3 g/L), followed by JI0%, JI1% and NI3% which were not significantly different ($p > 0.05$) (Fig. 1). Nevertheless, in fermented nectars, an addition of 3% inulin was required to reach the same survival values of *L. acidophilus* (7.76 Log CFU/mL) as in the juices without inulin (JI0%) or with 1% of inulin (JIN1%). The inulin consumption in NI3% was around 5 g/L (Fig. 1). In this period only the fermented samples without inulin (JIN0% and NI0%) had decreased monosaccharides concentration. Flimelová, Kňazovická, Čanigová, and Benczová (2013) incorporated *L. acidophilus* and 1% inulin into fresh cheese and showed that inulin addition did not improve *L. acidophilus* survival during 15 days at 7 °C with respect to control samples.

After 30 days of storage, *L. acidophilus* survival was higher in fermented juices than in fermented nectars regardless of the inulin concentration. During this period, the inulin concentration in JI3% continued to decrease (1 g/L), but without improving the viability of *L. acidophilus* compared to JI0% and JI1% (Table 3). However, inulin improved *L. acidophilus* viability in nectars, reaching 7.68 Log CFU/mL in NI1% and NI3%, while the lowest cell survival was observed in fermented nectars without inulin supplementation (NI0%) (7.61 Log CFU/mL). This suggests that inulin had a beneficial prebiotic effect on *L. acidophilus* viability during the first 30 days of storage.

At the end of storage, the *L. acidophilus* metabolism of sugars and inulin depended on the type of fermented beverage. The results showed that in the fermented juices with the highest inulin addition (JIN3%), the sucrose concentration and the TSS levels did not change during the storage period (Tables 1 and 2). However, these juices (JIN3%) had a decrease in monosaccharides ($p > 0.05$) compared to the previous levels at 30 days of storage, whereas in JIN1% the monosaccharides did not change significantly, showing an inulin reduction higher than JIN3% (4.40 and 1.78%,

respectively). Additionally, JIN0% had a remarkable increase in monosaccharides concentration, likely as a result of hydrolysis of sucrose. This increase suggests that during the last storage period, *L. acidophilus* stopped metabolizing which led to a reduction in probiotic viability. More studies with storage periods longer than 40 days are needed to test this hypothesis. Nevertheless, in the fermented nectar without inulin (N0%), a reduction of fructose content ($p > 0.05$) was observed compared to that at 30 days of storage. In NI1% only inulin was fermented (2.57%) by the *L. acidophilus*, while the sugar content remained unchanged after the last storage period compared to the results at 30 days. However, in NI3% a high decrease in sucrose content (4.11 g/L) and also a slight but significant fructose reduction was observed compared to 30 days. Despite the sugars and inulin being metabolized by *L. acidophilus* in the synbiotic beverages during the last storage period, the *L. acidophilus* viability remained unchanged compared to juices and nectars without inulin. Therefore, all beverages were a good medium to maintain the *L. acidophilus* survival above the recommended concentration (10^6–10^7 CFU/mL or g) (FAO/WHO, 2002) during the storage period of 40 days, irrespective of the inulin concentration.

With reference to the assessment of microbiological quality, yeasts and moulds were not detected in any sample (<10 CFU/mL) throughout the storage period. This indicates that the heat treatment of the samples was effective and there was adequate protection from contamination for at least 40 days at 4 °C.

3.2. Metabolism of organic acids during fermentation and storage

The organic acid concentrations of all juices and nectars after fermentation and during storage is shown in Table 4. Lactic acid was present in all fermented beverages after the fermentation period, resulting in an increase in the pH and TA values after fermentation and during storage compared to the initial values of 3.5 and 0.59 mg/L, respectively. However, the lactic acid concentration was different depending on the type of beverage, being higher in all fermented juices than in fermented nectars (around 2.5 g/L and 1.6 g/L respectively). At the same time, a decrease in malic acid was observed in all fermented beverages, with a higher loss in the fermented nectars (around 55% (w/v)) than in fermented juices (around 37% (w/v)). This may suggest that the malic acid was converted into lactic acid as a result of the malolactic fermentation by *L. acidophilus* (Table 4). The higher decarboxylation of malic acid in the nectars may be related to the lower consumption of sugars by *L. acidophilus* in these beverages as shown previously (Table 1). Acetic acid was not present in any of the fermented nectars and juices after fermentation and during the storage.

After 10 days of storage, a significant increase in lactic acid concentration was observed in all fermented juices, which remained stable until the end of the storage. However, lactic acid concentrations in nectars increased throughout storage, although the levels remained lower than in juices. After 20 days of storage, the fermented juices had a higher concentration of lactic acid than the fermented nectars (around 2.90 and 2.20 g/L, respectively). Furthermore, it was observed that citric acid also decreased throughout the storage period depending on the composition of beverages when compared to the initial concentration. The fermented juices without inulin (JI0%), had a high citric acid decrease (13.8%) during storage, as did synbiotic juices (approximately 15.5%). In the fermented nectars, the citric acid decrease was lower but significant (around 4%) during storage period with independence of the inulin concentration. Therefore, these results suggest that although citric acid was fermented by *L. acidophilus*, it did not become the main source of energy for *L. acidophilus* metabolism when compared to sugars and malic acid. However, other authors indicated that in pomegranate juice citric acid was the first carbon source for *L. acidophilus* when the sugars concentration was low (Mousavi, Mousavi, Razavi, Emam-Djomeh, & Kiani, 2010).

These changes in organic acid concentrations resulted in different pH and TA values during the storage period (Table 2). The pH values decreased throughout storage when compared to the initial values (pH of 3.5 in all beverages). At the end of storage period the pH values were in the range of 2.97–2.89 for fermented juices and 2.82–2.89 for fermented nectars. At the same time, in consonance with the pH decrease, the TA was shown to increase throughout the storage. The TA was higher in fermented juices

Table 4
Organic acids content in different formulations of juices and nectars.[a]

Acids	Storage time (days)	Formulations[b]							
		CJ	JI0%	JIN1%	JIN3%	CN	NI0%	NI1%	NI3%
Malic (g/L)	0	4.20 ± 0.01Aa	4.21 ± 0.01Ae	4.26 ± 0.65Ac	4.24 ± 0.45Ac	4.25 ± 0.00Ba	4.26 ± 0.02Bd	4.27 ± 0.10Bd	4.26 ± 0.01Be
	1 (AI)	4.21 ± 0.01Ca	2.64 ± 0.08Bd	3.30 ± 0.81Bbc	2.62 ± 0.71Bb	4.25 ± 0.01Ca	1.89 ± 0.00Ac	1.70 ± 0.00Ac	1.81 ± 0.01Ad
	10	4.21 ± 0.03Da	2.09 ± 0.39ABc	2.93 ± 0.46Cab	2.51 ± 0.29BCb	4.24 ± 0.04Da	1.86 ± 0.01Ab	1.66 ± 0.05Abc	1.67 ± 0.01Ac
	20	4.21 ± 0.17Fa	2.06 ± 0.13Dc	2.14 ± 0.01Ea	2.18 ± 0.14Da	4.25 ± 0.01Fa	1.82 ± 0.00Ca	1.60 ± 0.00Aab	1.63 ± 0.00Bb
	30	4.21 ± 0.01Ea	1.91 ± 0.18Cb	1.94 ± 0.36CDa	1.95 ± 0.01Da	4.25 ± 0.01Ea	1.81 ± 0.00Ba	1.58 ± 0.00Aa	1.61 ± 0.02Aab
	40	4.21 ± 0.01Da	1.80 ± 0.13Ba	1.87 ± 0.01Ba	2.06 ± 0.15Ca	4.25 ± 0.01Da	1.80 ± 0.00ABa	1.56 ± 0.00Aa	1.58 ± 0.01ABa
Citric (g/L)	0	9.35 ± 0.11Aa	9.35 ± 0.15Ad	9.35 ± 0.21Ac	9.38 ± 0.26Ad	9.38 ± 0.12Aa	9.38 ± 0.01Ad	9.38 ± 0.05Ae	9.40 ± 0.02Ae
	1 (AI)	9.38 ± 0.01Ba	8.44 ± 0.29Ac	8.54 ± 0.37Ab	8.50 ± 0.12Ac	9.38 ± 0.12Ba	9.25 ± 0.01Bd	9.30 ± 0.04Bd	9.36 ± 0.02Bde
	10	9.35 ± 0.11Ba	8.43 ± 0.56Abc	8.53 ± 0.16Ab	8.31 ± 0.02Ab	9.38 ± 0.19Ba	9.15 ± 0.21Bcd	9.25 ± 0.03Bcd	9.35 ± 0.04Bd
	20	9.38 ± 0.01Fa	8.13 ± 0.36Aabc	8.44 ± 0.38Cb	8.20 ± 0.03BAb	9.38 ± 0.18Fa	8.93 ± 0.03Dbc	9.18 ± 0.01Ec	9.23 ± 0.01Ec
	30	9.36 ± 0.24Da	8.08 ± 0.01Aab	7.98 ± 0.15Aa	7.96 ± 0.01Aa	9.37 ± 0.16Da	8.89 ± 0.10Bb	9.07 ± 0.01Cb	9.16 ± 0.01Cb
	40	9.36 ± 0.52Ea	8.06 ± 0.02Ba	7.89 ± 0.04Aa	7.87 ± 0.04Aa	9.37 ± 0.16Ea	8.65 ± 0.01Ca	8.97 ± 0.02Da	9.01 ± 0.01Da
Lactic (g/L)	0	0.00 ± 0.00Aa	0.00 ± 0.00Aa	0.00 ± 0.00Aa	0.00 ± 0.00Aa	0.00 ± 0.00Aa	0.00 ± 0.00Aa	0.00 ± 0.00Aa	0.00 ± 0.00Aa
	1 (AI)	0.00 ± 0.00Aa	2.51 ± 0.02Cb	2.53 ± 0.64Cb	2.48 ± 0.04Cb	0.00 ± 0.00Aa	1.63 ± 0.03Bb	1.66 ± 0.00Bb	1.66 ± 0.04Bb
	10	0.00 ± 0.00Aa	2.86 ± 0.10Dc	2.86 ± 0.05Ec	2.80 ± 0.03Cc	0.00 ± 0.00Aa	1.99 ± 0.00Bc	1.99 ± 0.01Bc	1.99 ± 0.02Bc
	20	0.00 ± 0.00Aa	2.91 ± 0.59Dd	2.86 ± 0.04Cc	2.82 ± 0.14Cd	0.00 ± 0.00Aa	2.15 ± 0.04Bd	2.13 ± 0.01Bd	2.13 ± 0.02Bd
	30	0.00 ± 0.00Aa	2.92 ± 0.06Gd	2.86 ± 0.03Fc	2.83 ± 0.06Ed	0.00 ± 0.00Aa	2.23 ± 0.02De	2.18 ± 0.01Ce	2.15 ± 0.03Be
	40	0.00 ± 0.00Aa	2.93 ± 0.03Dd	2.87 ± 0.49Cc	2.84 ± 0.03Cd	0.00 ± 0.00Aa	2.22 ± 0.02Be	2.21 ± 0.01Bf	2.18 ± 0.00Bf

AI, after incubation period (36 h at 37 °C).

[a] Means ± standard deviation in the same column followed by different lowercase letters indicate statistically significant differences at ($p \leq 0.05$) for each formulation affected by the storage time (n = 3). Means ± standard deviation in the same row followed by different uppercase letters indicate statistically significant differences at ($p \leq 0.05$) between formulations for the same storage time (n = 3).

[b] Formulations: CJ; unfermented control juices; JIN0%, juices without inulin; JIN1%, juices + 1% of inulin; JIN3%, juices + 3% of inulin; CN, unfermented control nectars; N0%, nectar without inulin; NI1%, nectar + 1% of inulin; NI3%, nectar + 3% of inulin.

(1.46–1.41% lactic acid) than in fermented nectars (1.31–1.25% lactic acid) at the end of storage period. Therefore, the extent of lactic acid production in all beverages depended on the chemical composition of the drinks that influenced the metabolism of the different sugars, inulin and malic acid. In a previous study, it was also reported that in cereal beverages (oat, barley and malt) fermented with *L. acidophilus*, the lactic acid concentration was different in oat compared to barley and malt beverages after the fermentation process (Salmerón, Thomas, & Pandiella, 2014, 2015).

3.3. Sensory acceptability of fermented juices and nectars after fermentation and during storage

The sensory acceptance tests of nectars and juices supplemented with different concentrations of inulin were conducted the first day after the fermentation and during the storage (at 20 and 40 days) (Table 5). At the beginning of storage, there was no significant difference in the acceptability of the colour and aroma between samples. The results for the acceptability of the colour were around 8 in a 9 point scale ($p > 0.05$) indicating that the consumers liked very much this attribute. For the aroma, the values were between 6.5 and 7.5 meaning that it was moderately liked. The beverage composition (juices or nectars) and inulin supplementation impacted the sweetness, acidity and flavour acceptance. Regarding the overall acceptance, the fermented nectars with inulin were the most accepted, while the control juices had the lowest acceptance. The fermented nectars supplemented with inulin scored the best in sweetness, acidity and flavour acceptance. The absence of acetic acid after fermentation in the drinks may result in a better acceptance, because this acid is related to distasteful flavour described as sour and vinegar (Salmerón et al., 2015).

The study of the sensory acceptance of the fermented juices and nectars by the consumers during storage is interesting, because of the continuous physicochemical changes (decreases in sugars concentration and pH and increase in acidity) that may contribute to a negative acceptability by the consumers (Pimentel, Madrona, et al., 2015b). During the storage period, the consumers indicated the lowest score in acidity acceptance for the fermented juices without inulin (JI0%), probably because of the highest perception of the acidity due to lactic acid in the absence of inulin. However, the fermented nectars with the highest inulin addition (NI3%) were the samples with the best score for acidity. These results suggest that supplementation with 3% inulin along with sucrose helped to reduce the perception of acidity. In other studies it has been observed that the use of sweeteners in apple juices suppressed the acidity perception (Pimentel, Madrona, et al., 2015b). At the same time, fermented nectars with inulin had higher sweetness acceptance values than fermented juices with inulin. This could be due to the presence of sucrose together with inulin. Therefore, although inulin has 35% of the sweetness of sucrose (Shoaib et al., 2016), the amount of inulin used in the supplementation of juices (1 and 3%) was not sufficient to contribute to the sweetness and reach the same acceptance values as in the nectars.

The nectars fortified with inulin were the most preferred ($p < 0.05$) during storage (20 and 40 days). Possibly, the high acceptance values in sweetness and acidity influenced the overall acceptance score in these beverages. Therefore, the substrate composition (more diluted components, sucrose and inulin addition) in nectars had a positive influence on the sensory acceptance. The other fermented beverages showed the same overall scores as the control samples (CJ and CN). The results indicate that the presence of *L. acidophilus* and the changes produced on the chemical composition in the beverages, did not have a significant impact in the acceptance of the fermented samples when compared with the control samples. In previous studies, inulin addition in dairy beverages improved the flavour and aroma attributes (Silveira et al., 2015) and ricotta fermentation with *L. acidophilus* showed satisfactory sensory results during storage (Meira et al., 2015). Other studies have reported that papaya nectar with inulin was more accepted than nectars supplemented only with sucrose (Braga & Conti-Silva, 2015). For this reason, the supplementation of vegetable drinks with prebiotic ingredients, such

Table 5
Acceptability of different formulations of juices and nectars.[a]

Parameters	Storage time (days)	Formulations[b]							
		CJ	JI0%	JIN1%	JIN3%	CN	NI0%	NI1%	NI3%
Colour	1 (AI)	7.31 ± 1.58Aa	7.37 ± 1.63Aa	7.31 ± 1.25Ab	7.37 ± 1.50Aa	7.75 ± 1.29Aa	7.81 ± 1.22Aa	8.00 ± 1.09Aa	8.06 ± 0.99Aa
	20	7.75 ± 1.48Aa	7.75 ± 1.24Aa	7.31 ± 1.14Aa	7.75 ± 1.48Aa	7.87 ± 1.36Aa	7.87 ± 1.09Aa	7.78 ± 1.09Aa	7.81 ± 1.38Aa
	40	7.37 ± 1.02Aa	7.56 ± 1.26Aa	7.35 ± 1.29Aa	7.75 ± 0.77Aa	7.50 ± 1.59Aa	7.50 ± 1.79Aa	7.37 ± 1.67Aa	7.87 ± 1.50Aa
Aroma	1 (AI)	6.37 ± 1.54Aa	7.12 ± 1.45Aa	7.25 ± 1.29Ab	6.93 ± 1.61Aa	7.25 ± 1.69Ab	6.50 ± 1.67Aa	7.62 ± 1.66Ab	7.75 ± 1.69Aa
	20	5.37 ± 1.15Aa	6.31 ± 2.30ABa	6.00 ± 1.67ABa	5.87 ± 1.89ABa	6.81 ± 1.22Bab	6.37 ± 1.26ABa	6.44 ± 1.34ABab	6.75 ± 1.41Ba
	40	5.25 ± 1.70Aa	5.87 ± 1.89ABa	5.87 ± 1.59ABa	6.25 ± 1.06ABa	6.06 ± 1.48ABa	5.94 ± 1.77ABa	6.37 ± 1.36ABa	6.69 ± 1.45Ba
Sweetness	AF	5.69 ± 1.99Aa	6.31 ± 1.74ABa	6.19 ± 0.91ABa	6.56 ± 1.67ABa	6.37 ± 1.89ABa	6.88 ± 1.88ABa	7.12 ± 1.66Bb	7.37 ± 1.45Ba
	20	5.69 ± 2.12Aa	5.12 ± 1.78Aa	5.62 ± 1.86Aa	5.81 ± 1.33Aa	6.56 ± 1.09Ba	6.12 ± 1.19ABa	6.37 ± 1.45 Ba	6.69 ± 1.59Ba
	40	5.37 ± 1.67Aa	5.62 ± 2.09Aa	5.50 ± 1.15Aa	5.75 ± 1.18Aa	6.00 ± 1.31Aa	5.75 ± 2.01Aa	6.37 ± 1.67Ba	6.65 ± 1.54Ba
Acidity	1(AI)	6.81 ± 1.64ABb	5.81 ± 2.07Ab	6.50 ± 1.09ABb	6.56 ± 1.63ABb	6.44 ± 1.96ABa	6.62 ± 1.78ABa	7.43 ± 1.78ABb	7.00 ± 1.71Ba
	20	5.06 ± 2.17ABa	4.62 ± 1.86Aa	5.56 ± 1.15ABCab	5.81 ± 1.22ABCa	6.19 ± 1.97BCa	6.44 ± 2.06BCa	6.56 ± 1.59BCab	6.62 ± 1.99Ca
	40	5.00 ± 2.16ABa	4.37 ± 1.09Aa	5.37 ± 2.03ABa	5.87 ± 1.54ABa	5.81 ± 1.38ABa	5.56 ± 1.41ABa	5.43 ± 1.69ABa	5.75 ± 1.77Ba
Flavour	1	5.75 ± 2.26Aa	6.37 ± 1.63ABCb	6.56 ± 1.26ABCb	6.87 ± 1.50ABCb	6.12 ± 1.89ABCa	6.94 ± 1.84ABb	7.19 ± 1.54BCb	7.37 ± 1.76Ca
	20	5.75 ± 1.73ABa	5.25 ± 1.57Aa	5.62 ± 1.89ABab	5.87 ± 1.59ABCab	6.00 ± 1.32BCa	5.75 ± 1.27ABCab	6.81 ± 1.52Ca	6.69 ± 1.25Ca
	40	5.12 ± 1.89Aa	5.00 ± 2.16Aa	5.37 ± 1.41Aa	5.81 ± 1.11Aa	5.62 ± 1.45Aa	5.56 ± 1.71Aa	5.68 ± 1.30Aa	5.94 ± 1.69Aa
Overall acceptance	1(AI)	5.81 ± 1.90Aa	7.00 ± 1.71ABb	6.94 ± 1.23ABb	7.06 ± 1.65ABb	6.87 ± 1.86ABa	6.50 ± 1.96ABb	7.25 ± 1.78Bb	7.62 ± 1.52Ba
	20	5.12 ± 1.86Aa	5.00 ± 1.67Aa	5.44 ± 1.55Aa	5.44 ± 1.41ABa	6.44 ± 1.63BCa	5.68 ± 1.67BCa	6.43 ± 1.57BCa	6.75 ± 1.57Ca
	40	5.19 ± 1.83Aa	5.12 ± 2.19Aa	5.56 ± 1.59Aa	5.62 ± 1.36Aa	5.81 ± 1.32Aa	5.94 ± 1.39Aa	6.00 ± 1.25ABa	6.37 ± 1.70Ba

Hedonic values (colour, aroma, sweetness, acidity, flavor and overall acceptance: 1- dislike very much; 9 - like very much.
AF, after incubation period (36 h at 37 °C).
[a] Means ± standard deviation in the same column followed by different lowercase letters indicate statistically significant differences at ($p \leq 0.05$) for each formulation affected by the storage time (n = 3). Means ± standard deviation in the same row followed by different uppercase letters indicate statistically significant differences at ($p \leq 0.05$) between formulations for the same storage time (n = 3).
[b] Formulations: CJ; control juices; JIN0%, juices without inulin; JIN1%, juices + 1% of inulin; JIN3%, juices + 3% of inulin; CN, control nectars; N0%, nectar without inulin; NI1%, nectar + 1% of inulin; NI3%, nectar + 3% of inulin.

as inulin, can promote the growth of the *L. acidophilus* improving at the same time the sweetness and flavour.

4. Conclusions

In this study, carrot-orange juices and nectars were demonstrated to be good media to maintain *L. acidophilus* viability above the minimum recommended (10^6–10^7 CFU/mL) during storage. However, *L. acidophilus* metabolism was strongly dependent on the beverage composition. Inulin was used as a prebiotic in all beverages but it improved *L. acidophilus* viability in the nectar. The different ingredients added to the nectars (water and sucrose) together with inulin contributed to the viability being the beverage that was preferred by the consumers. Therefore, the present study represents an important contribution to the future development of healthier fruit and vegetable beverages, showing a good alternative to the traditional probiotic dairy products through the development of synbiotic nectars with three important properties: high *L. acidophilus* viability, good consumer acceptance and long shelf life.

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PUBLICATION 5

Influence of fermentation with different lactic acid bacteria and *in vitro* digestion on the biotransformation of phenolic compounds in fermented pomegranate juices

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Influence of Fermentation with Different Lactic Acid Bacteria and in Vitro Digestion on the Biotransformation of Phenolic Compounds in Fermented Pomegranate Juices

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S Supporting Information

ABSTRACT: This study describes the effect of fermentation and the impact of simulated gastrointestinal digestion (SGD) of four fermented pomegranate juices with different lactic acid bacteria (LAB) on the biotransformation of phenolic compounds. The changes of the antioxidant capacity (AOC) and of LAB growth and survival in different fermented juices were also studied. Two new phenolic derivatives (catechin and α-punicalagin) were identified only in fermented juices. During SGD, the AOC increased together with the phenolic derivatives concentration mainly in the juices fermented with *Lactobacillus*. These derivatives were formed due to the LAB metabolism of the ellagitannins, epicatechin, and catechin after fermentation and during SGD. The FRAP assay performance might be associated with the degradation and biotransformation of catechin. The fermented pomegranate juices with these LAB increased the bioaccessibility of phenolic compounds, ensuring the survival of LAB after SGD, suggesting a possible prebiotic effect of phenolic compounds on LAB.

KEYWORDS: *Lactobacillus, Bifidobacterium, antioxidant capacity, probiotic, bioactive compounds*

INTRODUCTION

Polyphenols are part of our diet and exert antioxidant properties; the main dietary sources are wines, fruits, juices, legumes, and vegetables.[1] The bioaccessibility of some polyphenols is very low because of their degree of polymerization and glycosylation pattern.[2] Therefore, their physiological benefits depend on the quantity of phenolic compounds that are available (bioavailability) to be absorbed in the intestine.[3,4] This suggests that a large number of phenolic compounds reach the colon, and due to the action of gut microbiota, these compounds are transformed, leading to the production of metabolites that can be absorbed, producing a physiological effect.[2,5]

Curiously, everybody has an individual microbiota composition, so that the bioavailability of polyphenols for the production of microbial metabolites is subjected to interindividual variability. Therefore, the health effects are not the same for everyone.[2,6,7]

In recent years, pomegranate juice has been investigated due to its beneficial properties because of its elevated concentration of polyphenols.[8] Those polyphenols include mainly anthocyanins, procyanidins, phenolic acids, flavonol glycosides, and hydrolyzable tannins such as ellagitannins, gallotannins, and punicalagins.[9] However, humans cannot absorb those hydrolyzable tannins and, therefore, these compounds are hydrolyzed to ellagic acid, which is also poorly absorbed, being metabolized in the colon by the microbiota to produce urolithins.[10] The urolithins together with other phenolic metabolites are mainly responsible for the health properties.[9] The capacity of lactic acid bacteria (LAB) to metabolize the phenolic compounds depends on the species or on the strain.[11,12] The microbial conversion of polyphenols can occur during a food fermentation process by LAB.[12−14] Those bacteria are the main source of probiotics, which are "live microorganisms that, when administered in adequate amounts confer health benefits on the host".[15] Although health beneficial effects due to *Lactobacillus* and *Bifidobacterium* have been shown, their function as antioxidants has not been fully investigated.[16]

The aim of this study was to investigate the biotransformation of the phenolic compounds in pomegranate juices (a) after fermentation by four LAB and (b) during in vitro digestion of unfermented and fermented juices. The antioxidant properties and LAB survival were also investigated in different fermented pomegranate juices to study the influence of fermentation and in vitro digestion on the polyphenol biotransformations.

MATERIALS AND METHODS

Activated Bacterial Strains and Culture Preparations. *Lactobacillus acidophilus* CECT 903 (LA), *Lactobacillus plantarum* CECT 220 (LP), *Bifidobacterium longum* subsp. *infantis* CECT 4551 (BL), and *Bifidobacterium bifidum* CECT 870 (BB) were purchased from the Spanish Type Culture Collection (CECT, Valencia, Spain) in lyophilized form. To obtain the pre-inoculum, each strain was resuspended in 10 mL of Man−Rogosa−Sharpe (MRS) broth (Oxoid, Madrid, Spain) at 37 °C during 24 h under aerobic conditions for *Lactobacillus* strains and during 48 h under anaerobic conditions for *Bifidobacterium* strains. After this time, to obtain an initial biomass of about 8 log colony-forming units per milliliter (CFU/mL), 1 mL of

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each pre-inoculum was inoculated in 100 mL of MRS broth and incubated for 24−48 h at 37 °C. The cultures were separated by centrifugation at 2000*g* for 10 min at 4 °C, washed twice with sterile phosphate buffer saline (PBS), and stored with glycerol at −80 °C until used.

Fermented Pomegranate Juices. Pomegranate juices in aseptic bags of 4.5 kg were provided by Probelte Biotecnología, S.L. (Murcia, Spain). According to the supplier's certificate of analysis, the pomegranate juice had 14.9% of soluble solids content, pH 3.7, and an acidity of 0.31 g citric acid/100 mL, as well as compliance with the microbiological criteria specified (total plate count, yeast, and mold, *Salmonella* sp. and *Escherichia coli*).

The pomegranate juices were put into sterile borosilicate glass bottles (250 mL) with polypropylene screw caps. Each pomegranate juice bottle (250 mL) was inoculated with 1% (v/v) of individual LAB previously prepared and incubated at 37 °C for 24 h to obtain four different fermented pomegranate juices (FPJ) with an initial concentration of 6 log CFU/mL: FPJ with *B. bifidum* (FPJBB), FPJ with *L. plantarum* (FPJLP), FPJ with *B. longum* subsp. *infantis* (FPJBL), and FPJ with *L. acidophilus* (FPJLA). The fermented juices were compared with two different unfermented controls: control juices with incubated at 37 °C (CPJI) or stored under refrigerated conditions at 4 °C (CPJR). The fermented pomegranate juices were analyzed after the incubation period together with the unfermented control juices.

Simulated Gastrointestinal Digestion. The in vitro digestion assay was carried out according to the procedure of Valero-Cases and Frutos[17] with some modifications. For the assays, 100 mL of each juice was subjected to simulated gastrointestinal digestion for 180 min at 37 °C under stirring. The simulated gastric juices (SGJ) were prepared with 400 mL of PBS to pH 3 with 1 M HCl (Panreac, Barcelona, Spain), and pepsin was added to reach a concentration of 3 g/L (Oxoid). After 60 min of gastric digestion, the simulated intestinal juices (SIJ) were prepared by increasing the pH to7 with 1 M $NaHCO_3$ (Panreac) and adding 4.5 g/L of bile salts and 1 g/L of pancreatin (Sigma, Madrid, Spain), and they were incubated during 120 min at 37 °C. Samples were taken in each of the fermented and control juices after incubation time and at the different stages of in vitro digestion: after 60 min of SGJ, after 60 min of SIJ (SIJ1), and after 120 min of SIJ (SIJ2).

Microbiological Analysis. The growth and survival of LAB after fermentation and during in vitro digestion were determined by plate count. Samples (10 mL) of each FPJ were taken after fermentation and after different steps of in vitro digestion (SGJ, SIJ1, and SIJ2). Suitable dilutions (0.1 mL) were spread in triplicate on MRS agar plates and incubated for 24−48 h at 37 °C under aerobic and anaerobic conditions depending on the LAB. The results were expressed as log CFU/mL of fermented pomegranate juice or SGJ or SIJ.

Extract Preparation. For the determination of the phenolic compounds and the antioxidant activity after the incubation time, the pomegranate juice extracts were prepared as previously described by Nuncio-Jáuregui et al.[18] with least modifications: all different juices (5 mL) were mixed with 10 mL of MeOH/water (75:25 v/v) with 0.1% HCl using an Ultra-Turrax (T25, IKA, China) for 5 min and centrifuged at 15000*g* for 15 min at 4 °C. The control samples stored at 4 °C followed the same extraction process. The supernatants were filtered (0.45 μm, Millipore; Spain) and stored at −80 °C until analysis. Samples from in vitro gastrointestinal digestion (10 mL) were taken directly at every digestion step, centrifuged, and stored following the same procedure described above.

HPLC-DAD Analysis (Identification and Quantification of Phenolic Compounds). The identification and quantification of phenolic compounds were done following the Robles-Sánchez et al.[19] method with some adaptations on the binary gradient elution system. High-performance liquid chromatography studies were performed using an Agilent 1200 series HPLC system (Agilent Technologies, Waldbronn, Germany) coupled with a diode array detector (DAD) equipped with a reversed-phase column C18 Waters Spherisorb ODS-1 (250 mm × 4.6 mm, 5 μm particle size, Mediterranea SEA18; Teknokroma S.C.L., Barcelona, Spain). A binary gradient elution system was composed of solvent A, deionized water with 1% formic acid, and solvent B, acetonitrile with 1% formic acid. The elution profile was as follows: 95% (A) at 0 min, 87% (A) at 15 min, 85% (A) at 20 min, and 70% (A) at 25 min and continued isocratically for 3 min, then changing to 55% (A) at 32 min and continued isocratically for 3 min, then changing to 10% (A) at 40 min and continued isocratically for 5 min, and then changing to 95% (A) at 60 min. The flow rate was 1 mL/min with an injection volume of 20 μL, and the temperature of the column was kept at 30 °C. Different pure standards, ellagic acid, α-punicalagin, β-punicalagin, punicalin, catechin, epicatechin, and galic acid (Sigma), were used to prepare different calibration curves. The standards were dissolved in MeOH/water (75:25 v/v) and acidified with 1% HCl. The chromatograms were carried out simultaneously at 260, 280, 320, 360, or 520 nm. The identification of phenolic compounds was carried out by comparing the retention time and UV absorption spectra with those of the standards and quantified using calibration curves of the standards. Microbial metabolites such as ellagic acid derivative, α-punicalagin derivative, and catechin derivative were identified according to the literature[20,21] by comparing the UV absorption spectrum with those of the standards and tentatively quantified using the calibration curves of ellagic acid, α-punicalagin, and catechin. All determinations were made in triplicate, and the results were expressed as milligrams per 100 mL.

Antioxidant Capacity Determined by Using the 2,2-Diphenyl-1-picrylhydrazyl (DPPH) Free Radical Scavenging Method. The free radical scavenging activity was determined using the DPPH method adopted from Brand-Williams et al.[22] Each supernatant (10 μL) was mixed with 40 μL of MeOH and added to 950 μL of DPPH solution. The mixture was shaken and kept during 10 min in a dark room. The absorbance decrease was measured at 515 nm in an UV−vis Uvikon XS spectrophotometer (Bio-Tek Instruments, Saint Quentin Yvelines, France). The calibration curve was of the form $y = 0.2443x + 0.0047$ ($R^2 = 0.999$) and was made using Trolox as standard solution in the range of 0.01−5.00 mmol/L. The analyses were run in three replications, and the results were expressed as millimoles of Trolox per liter of juice.

Antioxidant Capacity Determined by Using the Ferric Reducing Antioxidant Power (FRAP) Method. The FRAP method adopted from Benzie and Strain[23] was employed. Briefly, the FRAP reagent was prepared fresh daily by mixing 300 mmol/L acetate buffer (pH 3.6), 10 mmol/L TPTZ solution, in 40 mmol/L HCl, and 20 mmol/L $FeCl_3 \cdot 6H_2O$ solution in a volume ratio of 10:1:1, respectively. For the assays, 10 μL of each extract was mixed with 990 μL of FRAP and kept in a dark room for 10 min at 37 °C. Then, the absorbance was measured at 593 nm. The calibration curve was of the form $y = 0.4043x + 0.0626$ ($R^2 = 0.998$) using Trolox as standard solution in the range of 0.01−5.00 mmol/L. The analyses were run in three replications, and the results were expressed as millimoles of Trolox per liter of juice.

Antioxidant Capacity Determined by Using the 2,2′-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) Radical Scavenging Method. The ABTS radical cation method was measured adopted from the method developed by Re et al.[24] Briefly, the $ABTS^{\bullet+}$ was prepared by mixing 7 mM ABTS with 2.45 mM $K_2S_2O_8$, and this mixture was allowed to react for 12−16 h in the dark at room temperature. The solution was then diluted with PBS at pH 7.4 to an absorbance of 0.70 ± 0.02 at 734 nm. For the assays, 10 μL of each extract was mixed with 990 μL of ABTS and kept in a dark room for 10 min. The calibration curve was of the form $y = 0.2238x + 0.0322$ ($R^2 = 0.991$) using Trolox as standard solution in the range of 0.01−5.00 mmol/L. The analyses were run in three replications, and the results were expressed as millimoles of Trolox per liter of juice.

Statistical Analysis. All of the experiments and analyses were performed in triplicate. The results were expressed as the mean ± standard deviation. The mean comparison was performed via the SPSS v 21.0 software package (SPSS Inc., Chicago, IL, USA) using analysis of variance (ANOVA) followed by a Tukey multiple-range test to evaluate the significant differences ($p < 0.05$).

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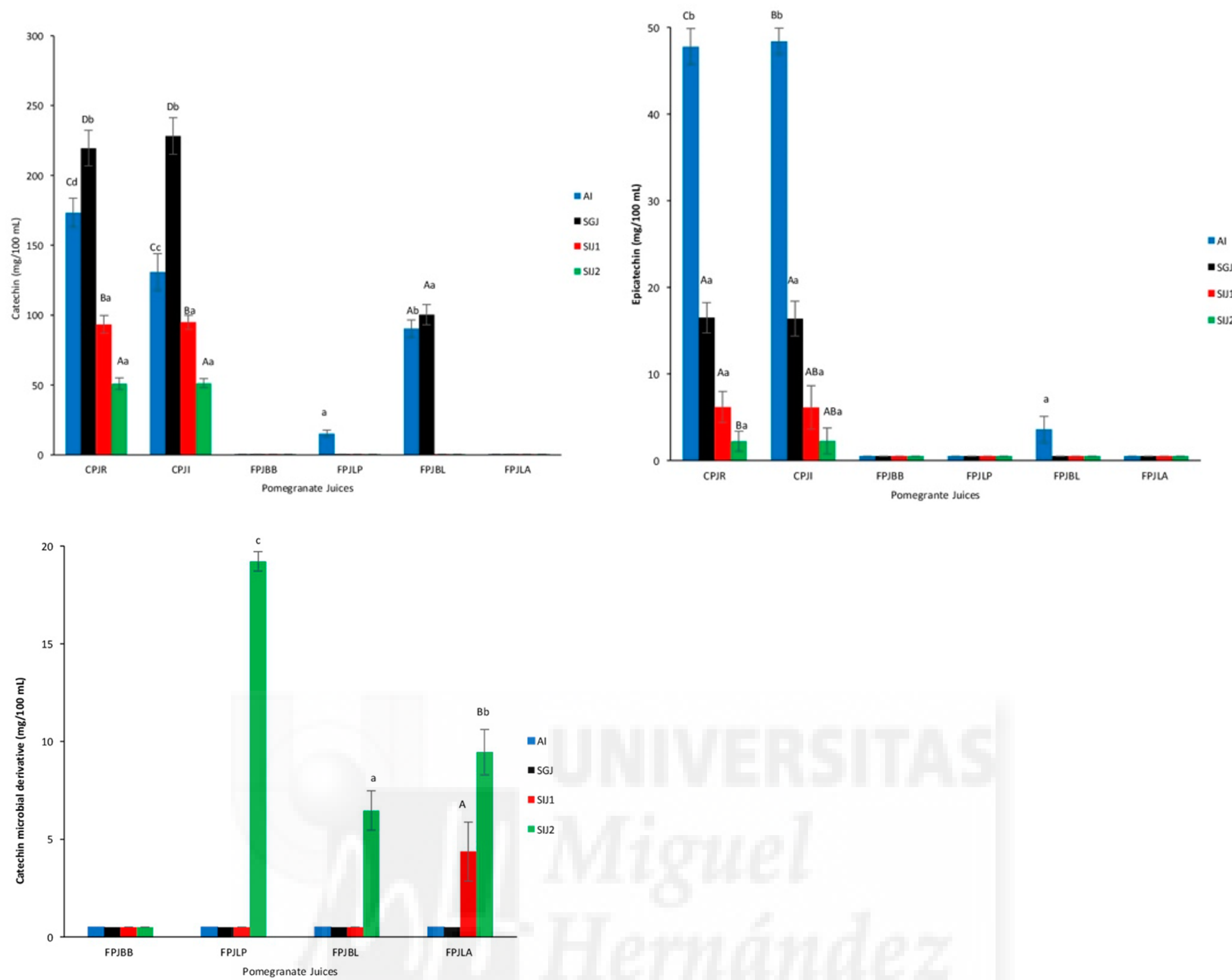

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Figure 1. Evolution of flavan-3-ol after incubation period and during the different steps of the in vitro digestion in different fermented pomegranate juices. The bars are the mean of three replications (±standard error). Capital letters refer to the flavan-3-ol evolution: after the incubation period (AI), simulated gastric juice (SGJ), simulated intestinal juices after 60 min (SIJ1), and simulated intestinal juices after 120 min (SIJ2) for the same type of juice. Lower case letters refer to the comparison among pomegranate juices for the same step: control pomegranate juice refrigerated (CPJR), control pomegranate juice incubated (CPJI), fermented pomegranate juice with *Bifidobacterium bifidum* (FPJBB), *Lactobacillus plantarum* (FPJLP), *Bifidobacterium longum* subsp. *infantis* (FPJBL), and *Lactobacillus acidophilus* (FPJLA). AI refers to the results after the incubation period for CPJI, FPJBB, FPJLP, FPJBL, and FPJLA at 37 °C compared to CPJR that was not incubated and remained refrigerated at 4 °C.

RESULTS AND DISCUSSION

Biotransformation of Phenolic Compounds in Fermented Pomegranate Juices after 24 h of Incubation. Figures 1 and 2 show the biotransformation of phenolic compounds by different lactic acid bacteria in pomegranate juices in relation with the control juices (CPJR and CPJI). In the control juices stored at different temperatures (4 and 37 °C), eight different phenolic compounds were identified: catechin, α- and β-punicalagin, punicalin, epicatechin, gallic acid, ellagic acid derivative, and ellagic acid. To our knowledge, all of these phenolic compounds have been reported in pomegranate juices in previous studies.[21,25−27] Most of the initial phenolic compound values did not show significant differences between the control samples stored at different temperatures (4 and 37 °C, CPJR and CPJI, respectively). However, it seems that when the control juices were stored at 37 °C, the catechin content was lower with respect to the initial concentration in control juices stored at 4 °C (130.87 vs 173.42 mg/100 mL, respectively) (Figure 1). At the same time, the microbial fermentation had an increase in the levels of the phenolic compounds; in the different experimental fermented juices, a total of nine compounds were identified in this study after fermentation (37 °C during 24 h). Eight of the phenolic compounds were the same as in the control juices. However, a new compound was found only in fermented pomegranate juices. This new phenolic metabolite was a new catechin derivative identified after the fermentation (by comparison of the spectral data with the standard) with respect to the control juices (Figure 1). Epicatechin was almost completely metabolized (only traces were obtained) by BB, LP, and LA and, to a lesser extent, by BL (3.59 mg/100 mL) (Figure 1). Catechin was completely degraded by BB and LA (only traces were obtained) and by LP and BL to a lesser extent (15.30 and 90.28 mg/100 mL were obtained, respectively). However, in control juices (CPJR and CPJI), the amounts of epicatechin and catechin were higher than in the fermented juices (ca. 48 and 130 mg/100 mL, respectively) (Figure 1). Therefore, this microbial-derived catechin could be synthesized from the

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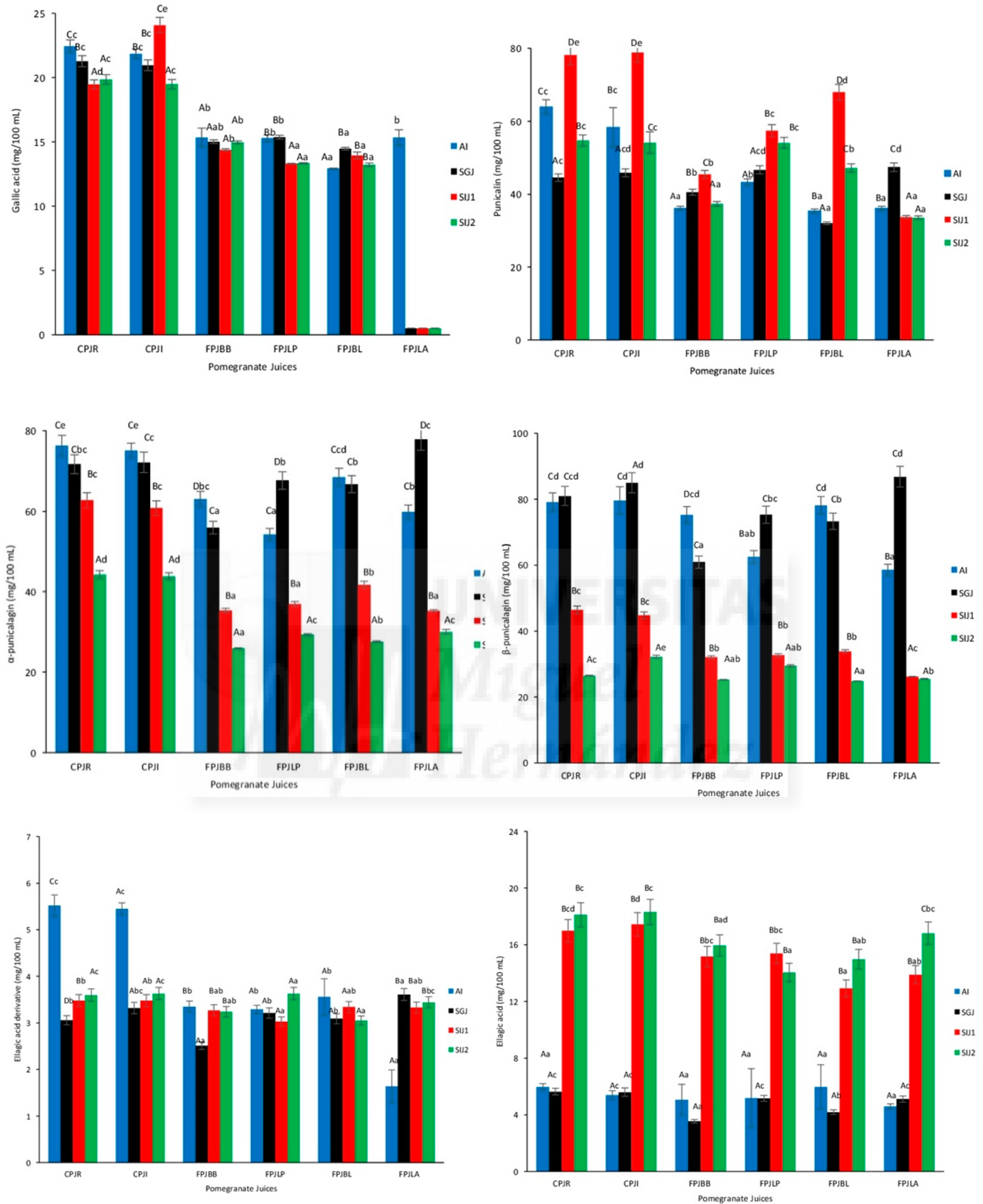

Figure 2. continued

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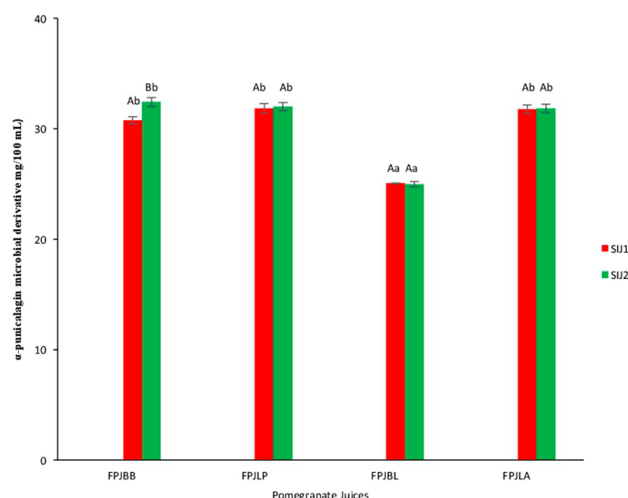

Figure 2. Ellagitannin evolution after the incubation period and during in vitro digestion in different fermented pomegranate juices. The bars are the mean of three replications (±standard error). Capital letters refer to the ellagitannin evolution: after incubation period (AI), simulated gastric juice (SGJ), simulated intestinal juices after 60 min (SIJ1), and simulated intestinal juices after 120 min (SIJ2) for the same type of juice. Lower case letters refer to the comparison among pomegranate juices for the same step: control pomegranate juice refrigerated (CPJR), control pomegranate juice incubated (CPJI), fermented pomegranate juice with *Bifidobacterium bifidum* (FPJBB), *Lactobacillus plantarum* (FPJLP), *Bifidobacterium longum* subsp. *infantis* (FPJBL), and *Lactobacillus acidophilus* (FPJLA). AI refers to the results after the incubation period for CPJI, FPJBB, FPJLP, FPJBL, and FPJLA at 37 °C compared to CPJR that was not incubated and remained refrigerated at 4 °C.

Table 1. Growth of Different Lactic Acid Bacteria in Pomegranate Juices after the Incubation Period and Their Survival during the Different Steps of the in Vitro Digestion[a]

	log CFU/mL			
period	FPJBB	FPJLP	FPJBL	FPJLA
AI	7.78 ± 0.05Da	7.33 ± 0.02Ca	7.67 ± 0.02Da	7.26 ± 0.07Ba
SGJ	7.40 ± 0.01Cc	6.83 ± 0.04Ba	7.18 ± 0.01Cb	6.93 ± 0.02Aa
SIJ1	7.09 ± 0.01Bc	6.73 ± 0.02ABa	7.11 ± 0.01Bc	6.88 ± 0.01Ab
SIJ2	6.79 ± 0.15Ab	6.71 ± 0.02Aa	6.99 ± 0.02Ac	6.82 ± 0.01Ab

[a]Values are the mean of three replications (±standard error). Capital letters refer to the evolution of each period: after incubation (AI), simulated gastric juice (SGJ), simulated intestinal juices after 60 min (SIJ1), and simulated intestinal juices after 120 min (SIJ2). Lower case letters are the comparison among fermented pomegranate juices with *Bifidobacterium bifidum* (FPJBB), *Lactobacillus plantarum* (FPJLP), *Bifidobacterium longum* subsp. *infantis* (FPJBL), and *Lactobacillus acidophilus* (FPJLA). Values followed by the same letter within the same column or row were not statistically different according to Tukey's multiple-range test ($p < 0.05$).

metabolism of other phenolic compounds (epicatechin and catechin) during bacterial fermentation, due to the different concentrations in relation to the control samples. These results are in agreement with Alberto et al.,[20] who reported the identification of intermediate metabolites from catechin due to *Lactobacillus hilgardii* fermentation. Otherwise, a decrease of ca. 40% in the ellagic acid derivative in FPJBB, FPJLB, and FPJLP was observed with respect to the control juices (Figure 2). Nevertheless, in FPJLA the decrease was 70% higher with respect to the unfermented juices.

On the other hand, with respect to the β-punicalagin and α-punicalagin concentrations, in the pomegranate juices fermented by *Lactobacillus* (FPJLP and FPJLA) the concentrations of β- and α-punicalagin were lower than in the juices fermented by *Bifidobacterium* strains (FPJBB and FPJBL) (Figure 2). However, the punicalin remained without significant differences between all fermented juices, whereas the gallic acid concentrations in FPJBL were the lowest (12.94 mg/100 mL). The free ellagic acid concentrations in control and fermented juices did not change during the incubation time and hence did not present significant differences between juices (Figure 2). The low biotransformation of ellagic acid in this step might be due to its insolubility in aqueous media especially at low pH.[28,29] Previous studies with cherry juice and broccoli puree fermented only by different *Lactobacillus* strains also showed the biotransformation of phenolic compounds during fermentation.[12]

At the same time, the number of viable cells was determined (Table 1) to study the relationship between the viable cells concentration and the biotransformation of phenolic compounds. Despite pomegranate juices being a hostile ecosystem (pH, buffering capacity) and needing a long time for fermentation for the growth of LAB,[13] in the present study all strains increased from 6 to 7.26−7.78 log CFU/mL without significant differences ($p > 0.05$) among the LAB used. This growth increase could be in relation to the metabolism of most of the pomegranate phenolic compounds to a greater or lesser extent, depending on the strain as stated before. Previous studies in pomegranate juice fermented by LAB showed the same increase after 120 h at 30 °C (from ca. 7.0 to 7.3 log CFU/mL) and a higher one (8 log CFU/mL) after 72 h at 37 °C.[21−30] Hence, in this study good growth was observed with different LAB and lower fermentation times (24 h at 37 °C).

Metabolism and Biotransformation of Phenolic Compounds in Fermented Pomegranate Juices after Gastric Digestion. The phenolic compound concentrations

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Table 2. Antioxidant Capacity of Pomegranate Juice after the Incubation Period and during the Different Steps of the in Vitro Digestion[a]

		mmol Trolox/L					
	period	CPJR	CPJI	FPJBB	FPJLP	FPJBL	FPJLA
DPPH	AI	20.41 ± 0.59Cab	18.85 ± 1.40Ca	20.40 ± 0.95Aab	22.22 ± 1.05Abc	24.04 ± 0.75Ac	22.93 ± 0.23Ac
	SGJ	14.40 ± 1.27Ba	13.07 ± 1.33Ba	27.27 ± 0.25Bb	27.99 ± 0.60Bb	27.32 ± 0.19ABb	27.90 ± 0.45Cb
	SIJ1	7.17 ± 0.26Aa	9.79 ± 2.60ABa	27.22 ± 0.46Bb	26.50 ± 0.18Bb	27.08 ± 0.47Bb	26.32 ± 0.37Bb
	SIJ2	7.50 ± 0.33Aa	6.95 ± 0.74Aa	27.26 ± 0.37Bb	27.05 ± 1.13Bb	26.39 ± 0.42ABb	26.56 ± 0.49Bb
ABTS	AI	13.83 ± 1.16Ac	12.52 ± 0.15Aabc	11.97 ± 0.23Aab	12.87 ± 0.92Abc	11.88 ± 0.82Aab	10.67 ± 0.25Aa
	SGJ	14.66 ± 0.49Abc	14.03 ± 0.85Bd	15.41 ± 0.27Bd	15.74 ± 0.44Bd	13.23 ± 0.21Bab	12.08 ± 0.40ABa
	SIJ1	14.47 ± 0.36Abc	13.84 ± 0.40Babc	15.35 ± 0.50Bbc	14.56 ± 0.20Bc	13.52 ± 0.31Bab	12.48 ± 1.14Ba
	SIJ2	13.87 ± 0.27Aa	14.02 ± 0.60Ba	15.39 ± 0.40Bb	15.16 ± 0.68Bb	14.44 ± 0.45Bab	14.63 ± 0.53Cab
FRAP	AI	13.94 ± 0.56Ba	13.32 ± 0.39Ca	15.99 ± 1.46Cb	13.51 ± 0.58Ca	14.68 ± 0.91Cab	16.06 ± 1.35Cb
	SGJ	10.80 ± 0.51Aa	12.27 ± 0.24Ba	12.05 ± 0.72Ba	11.98 ± 0.99Ba	11.31 ± 1.41Ba	12.41 ± 0.16Ba
	SIJ1	10.15 ± 0.43Ab	10.16 ± 0.43Ab	9.10 ± 0.69Aab	8.58 ± 0.21Aa	8.63 ± 0.35Aa	8.99 ± 0.08Aa
	SIJ2	10.68 ± 0.53Ab	10.01 ± 0.04Ab	8.42 ± 0.18Ab	8.41 ± 0.24Ab	8.71 ± 0.13Ab	8.84 ± 0.32Ab

[a]Values are the mean of three replications (±standard error). Capital letters are the evolution of period: after incubation (AI), simulated gastric juice (SGJ), simulated intestinal juices after 60 min (SIJ1), and simulated intestinal juices after 120 min (SIJ2). Lower case letters are the comparison among pomegranate juices [unfermented control pomegranate juice refrigerated (CPJR), unfermented control pomegranate juice incubated (CPJI), and fermented pomegranate juice with *Bifidobacterium bifidum* (FPJBB), *Lactobacillus plantarum* (FPJLP), *Bifidobacterium longum* subsp. *Infantis* (FPJBL) and *Lactobacillus acidophilus* (FPJLA)]. Values followed by the same letter within the same column or row were not statistically different according to Tukey's multiple-range test ($p < 0.05$). AI refers to the results after incubation period for CPJI, FPJBB, FPJLP, FPJBL, and FPJLA compared to CPJR that were not incubated and remained at 4 °C as refrigerated control juices.

were measured with the aim of testing their stability after 60 min in SGJ in the different juices (Figures 1 and 2). The SGJ conditions promoted the degradation of the epicatechin in the control juices with an increase (ca. 30%) in the catechin concentration in these juices (Figure 1). With respect to the fermented juices, the α- and β-punicalagin concentrations increased (ca. 25 and 30%, respectively) in the juices fermented by *Lactobacillus* (FPJLA and FPJLP), and only in FPJLA was it found that the gallic acid was fully metabolized by LA after gastric digestion. The FPJBB presented the lowest concentrations of these compounds (56.00 and 60.92 mg/100 mL, respectively). A decrease in the concentration of ellagic acid (ca. 30%) in the juices fermented by *Bifidobacterium* (FPJBB and FPJBL) was also observed (Figure 2). At the same time, the survival in *Bifidobacterium* FPJs was higher than in the juices fermented by *Lactobacillus*, with concentrations of 7.40 and 7.18 log CFU/mL for BB and BL, respectively (Table 1). These survival differences could be due to a higher metabolism of phenolic compounds by *Bifidobacterium* strains during SGJ (Figures 1 and 2). In other studies with apples and blackberries, phenolic compounds such as flavonoids, lignans, and phenolic acids were stable to the gastric conditions.[31,32] However, in other studies with dried figs and pomegranate extracts, a slight decrease in such compounds was observed.[5,33] It has to be pointed out that none of the previous studies was performed with fermented food matrices.

Metabolism and Biotransformation of Phenolic Compounds in Fermented Pomegranate Juices during and after Intestinal Digestion. The different phenolic compounds were measured in all juices after 60 min (SIJ1) and after 120 min of intestinal digestion (SIJ2) to study and compare their metabolism and stability with AF and SGJ (Figures 1 and 2). After SIJ1, an increase of ellagic acid in all juices was observed, with amounts 3 times higher than those found after gastric digestion (Figure 2). The SIJ conditions (neutral pH, presence of pancreatic enzymes and bile salts) could promote the transformation of α- and β-punicalagins into ellagic acid.[34] Consequently, a decrease was observed in those phenolic compounds (α- and β-punicalagin), with a higher decrease (ca. 40%) of α-punicalagin content in the fermented juices with respect to the control juices (ca. 14%) (Figure 2). This decrease could be related to the generation of a new α-punicalagin derivative that was detected only in fermented juices as a possible metabolite of the microbial transformations. The concentrations in fermented juices with BB, LP, and LA were between 30 and 31 mg/100 mL, whereas the FPJBL presented a lower concentration (25.07 mg/100 mL).

On the other hand, after SIJ1, decreases of catechin and epicatechin in the control juices were observed (ca. 45 and 64%, respectively) (Figure 1). The decrease in control juices could be due to the instability of the catechin at neutral pH.[35−37] In this digestion step, these compounds (catechin and epicatechin) were not detected in fermented juices because of a previous biotransformation of these compounds due to the LAB metabolism. The catechin derivative was detected only in fermented juices with respect to the control juices, and it was present in higher concentrations (4.37 mg/100 mL) in FPJLA, whereas the other fermented juices presented only trace amounts of these compounds after SIJ1 (Figure 1). In different studies with green and black tea, an important decrease was also observed in catechin after intestinal digestion.[35,37]

After 120 min of SIJ, following the same pattern as in SIJ1, the phenolic compound metabolism observed in fermented juices was higher than in the control juices, probably because of the longer time in intestinal juices with the microorganisms. The catechin derivative (detected only in fermented juices) increased significantly in FPJLP, FPJBL, and FPJLA (from trace amounts to 19.22, 6.47, and 9.45 mg/100 mL, respectively), whereas in FPJBB only trace amounts were detected (Figure 1). However, the α-punicalagin derivative concentration in SPJ1 was stable during this same period (Figure 2). This fact may suggest that the bacterial metabolism of ellagitannins, catechin,

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and epicatechin occurs during all steps of the in vitro digestion, predominantly during the intestinal step. Generally, the decrease in phenolic compounds for this period was higher for the fermented juices with *Bifidobacterium* (FPJBB and FPJBL). At the same time, when the cell concentrations were compared between the LAB strains, it can be observed that the concentrations of the *Bifidobacterium* strains were higher than those of the *Lactobacillus* ones. The FPJBL presented the highest cell concentration at the end of in vitro digestion. This fact could be related to the higher metabolism of most of the phenolic compounds in these fermented juices with respect to the other fermented ones (Table 1). With regard to the cell concentration, our results showed that although the LAB survival in SIJ2 was lower than in the other digestion steps, the cell concentrations were high (>10^6 CFU/mL) after the entire in vitro digestion period. However, the lowest cell survival was observed for FPJLP, and the phenolic compound concentration in these juices was higher than in the other FPJs (Table 1). The relationship observed in fermented juices between the phenolic compounds and cell concentrations suggests the possible prebiotic effect of phenolic compounds on the LAB. The metabolites excreted by the LAB could produce health benefits through bioaccessibility or bioactivity even though they are not absorbed in the gut.[3] This is a preliminary study, and thus it could be considered as a basis for future studies to reinforce this hypothesis.

Effect of Fermentation and in Vitro Digestion on the Antioxidant Capacity of Pomegranate Juices. During the fermentation and gastrointestinal digestion, different transformations (epimerization, degradation, oxidation, and hydrolysis) may occur in the phenolic constituents of the pomegranate juices that could change the pattern (structure and levels) of their metabolites.[21,35] Therefore, it is necessary to use different methods for providing an estimate of the in vitro antioxidant capacity (AOC). In this study, three methods were used to evaluate the changes in the AOC after fermentation and during in vitro digestion. After fermentation, it was observed that the AOC was higher in the fermented samples for the three methods, depending on the AOC method used and on the bacterial strain (Table 2). Therefore, the DPPH scavenging activity of fermented juices with BL and LA was higher ($p < 0.05$) than the control samples, whereas the ABTS scavenging activity of fermented samples did not change with respect to the control juices. The FRAP values of FPJLA and FPJBB were higher ($p < 0.05$) than the control samples values. However, a continuous increase in the antioxidant capacity was observed in fermented samples during the gastric digestion period, for only the DPPH and ABTS assays. This increase in AOC for these assays could be due to the gastric conditions, with low pH and pepsin activity, that led to an improvement in the release of bioactive compounds such as epicatechin, catechin, and α-punicalagin (Figures 1 and 2) increasing their bioaccessibility and, thus, the interaction with LAB. Huang et al.[38] found the same results after chemical extraction and in vitro digestion in Chinese bayberry. These results are in agreement with those of Gullon et al.,[21] who found that the gastric digestion increased the inhibition values of the DPPH and ABTS in the pomegranate peel.

In the intestinal digestion, the increase observed in the AOC during the SGJ for the ABTS and DPPH methods remained without significant changes during the 2 h of intestinal digestion for all FPJs (Table 2). At the end of the in vitro digestion, the AOC for the DPPH and ABTS method was in most of the FPJs higher than in the control ones. Several authors have reported that the in vitro digestion has a high impact on the AOC measured with these methods, as can be demonstrated in the studies performed in 33 fruit types, where the inhibition of DPPH radical increased after in vitro digestion.[39] The results are also in agreement with those reported by Chandrasekara and Shahidi[4] and Wootton-Beard et al.,[40] who found an increase in the ABTS values after in vitro digestion for millet grain and for 23 vegetable juices, respectively.

Nevertheless, during in vitro digestion, the FRAP assay values, in contrast to the DPPH and ABTS assays, showed a decrease in AOC for all juices after the gastrointestinal step. However, this initial decrease in fermented samples was higher ($p \geq 0.05$) after 60 min under intestinal conditions. These values obtained after SIJ1 remained stable at the end of SIJ2 with values between 10.7 and 8.4 mmol Trolox/L. According to a previous study,[41] the FRAP assay values of the control, fermented, and digested juices under gastric conditions can be enhanced by the electron transfer reaction under acid pH conditions. Previous results obtained for catechin instability at neutral pH in control juices and by the metabolism of the LAB in fermented juices (Figures 1 and 2) could be of some relation with the results of the FRAP assay after SIJ1 (Table 2). A possible explanation could be related to the metal-chelating properties of catechin and epicatechin with an important contribution to the antioxidant activity of the juices.[42] Therefore, the degradation of catechin and epicatechin in control juices and the formation of the catechin microbial metabolite through LAB metabolism in fermented juices, could be associated with the reduction of the chelating activity, resulting in a lower antioxidant activity in the FRAP assay.[43,44]

In conclusion, the results of the present study showed that the fermentation of pomegranate juices improves the AOC evaluated by the DPPH and ABTS assays and modifies the type and amount of phenolic compounds with respect to the unfermented ones. Through the biotransformation of these compounds by LAB, two new phenolic derivatives were obtained (catechin and α-punicalagin). The in vitro digestion improved the AOC for the ABTS and DPPH assays. However, the FRAP assay was significantly influenced by the degradation and biotransformation of the catechin and epicatechin. At the same time, the pomegranate juices were a good food matrix to ensure a high viability (≥10^6 CFU/mL) of all LAB used in this study after in vitro digestion. Therefore, the LAB used in this study can transform the phenolic compounds present in pomegranate juices, suggesting a possible prebiotic effect of phenolic compounds.

Microbial metabolites derived from the fermentation of pomegranate juices and the high viability of microorganisms reaching the colon may contribute to the maintenance of gut health. Further research is needed to understand the mechanisms of action of phenolic microbial metabolites in humans.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.6b04854.

Representative HPLC chromatograms of fermented pomegranate juices and fermented pomegranate juices after gastrointestinal in vitro digestion (PDF)

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

AOC, antioxidant capacity; LAB, lactic acid bacteria; CFU, colony-forming units; PBS, phosphate buffer saline; CPJI, control pomegranate juices with incubation; CPJR, control pomegranate juices with refrigeration; FPJBB, fermented pomegranate juice with *Bifidobacterium bifidum*; FPJLP, fermented pomegranate juice with *Lactobacillus plantarum*; FPJBL, fermented pomegranate juice with *Bifidobacterium longum* subsp. *infantis*; FPJLA, fermented pomegranate juice with *Lactobacillus acidophilus*; SGD, simulated gastrointestinal human digestion; SGJ, simulated gastric juice; SIJ, simulated intestinal juices; SIJ1, simulated intestinal juices after 60 min; SIJ2, simulated intestinal juices after 120 min; AI, after incubation

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PUBLICATION 6

Influence of the fruit juices carriers on the ability of *Lactobacillus plantarum* DSM20205 to improve *in vitro* intestinal barrier integrity and its probiotic properties

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1 **Influence of the fruit juice carriers on the ability of *Lactobacillus plantarum***
2 **DSM20205 to improve *in vitro* intestinal barrier integrity and its probiotic**
3 **properties**

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ABSTRACT

The aim of present study was to investigate the influence of tomato and feijoa juices as fermentable carriers of *Lactobacillus plantarum* (LP DSM20205) on the *in vitro* ability of the bacterium to improve intestinal barrier function using the trans-epithelial electrical resistance (TEER) assay in an apical anaerobic model. The survival of LP DSM20205 in different fruit juices during *in vitro* digestion, as well as its adhesion capacity and potential cytotoxic effect on Caco-2 cells were also studied. The results showed that carrier fruit juices have a significant influence on LP DSM20205 growth, survival during *in vitro* digestion, adhesion capacity and TEER. All fermented samples were not cytotoxic to Caco-2 cells. The largest improvement intestinal barrier integrity was observed with fermented tomato juice. The digested fermented juices did not increase TEER, even though the LP DSM20205 in these samples adhered well. Therefore, LP DSM20205 has potential to be used as a probiotic in the production of fermented tomato and feijoa juices.

Keywords: Probiotic, gut function, lactic acid bacteria, epithelial cells, non-dairy products

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52 **INTRODUCTION**

53 Probiotics are defined as "live microorganisms which when administered in adequate
54 amounts, confer health benefits to the host". [1] There are essential aspects to be
55 considered when evaluating the efficacy of probiotic strains, such as their survival
56 during gastrointestinal digestion, adherence to intestinal epithelial cells and ability to
57 enhance intestinal barrier function.[2] The probiotics present in commercial food products
58 are mainly strains of *Lactobacillus* and *Bifidobacterium* species.[3] The majority of these
59 were isolated from animal or human sources, because these strains are thought to better
60 adapt to human intestinal conditions.[4] There are few studies determining the functional
61 properties of probiotic lactic acid bacteria isolated from vegetable sources.

62 The functional properties of probiotics can change depending on the physicochemical
63 properties of the carrier foods and ingredients used.[5] To date, most probiotic foods in
64 the market are fermented dairy products, such as milk and yoghurt.[6] Much of the
65 research to evaluate the *in vitro* digestion tolerance and adhesion ability of probiotic
66 bacteria has been carried out using milk foods as the carrier matrix to delivery
67 probiotics.[5, 7-8] These products present limitations for certain segments of the
68 population, such as consumers with dairy allergies or lactose intolerance, or those with
69 cholesterolemia.[9]

70 Fruit juice could be used as an alternative fermentable carrier for probiotics. Fruit drinks
71 are an excellent source of antioxidant vitamins, bioactive compounds and minerals.
72 Furthermore, they have attractive flavours and are refreshing.[10-11] These nutritional and
73 sensory properties can be enhanced by fermenting these beverages with probiotic
74 microorganisms and, at the same time, fermentation helps to improve the juice shelf-life

75 [12]. Currently there is a lack knowledge about the effect of fruit juice carriers on the
76 activity and survival of probiotic bacteria.[9]
77 The hypothesis of this research was that fruit juices are a suitable carrier for probiotics
78 and that they enhance the probiotic's ability to improve intestinal barrier function. For
79 this study, two exemplar fruit juices were selected; feijoa (*Feijoa sellowiana*,
80 Myrtaceae) and tomato (*Lycopersicon esculentum)*. Feijoa is a highly aromatic fruit
81 grown widely in New Zealand from March until June. It is an good source of vitamin C
82 and minerals, low in calories with excellent antioxidant properties.[13] In contrast, tomato
83 (*Lycopersicon esculentum)* is a fruit widely grown worldwide and is a good source of
84 vitamins A and C, phosphorus and iron.[14] A probiotic strain isolated from a vegetable
85 source (*Lactobacillus plantarum* DSMZ20205 isolated from corn silage) was selected
86 for this study because it more likely to favour a fruit juice carrier than animal or human
87 derived probiotics.
88 To test our hypothesis, Caco-2 cell monolayers (a human intestinal epithelial cell line)
89 were grown in a novel dual-environment co-culture system that was used inside an
90 anaerobic workstation.[15] The apical (luminal) side of the Caco-2 cell monolayers were
91 maintained in an anaerobic environment to allow LP DSM20205 to have a similar
92 metabolism to what would occur in the (anaerobic) colon. In contrast, the bottom
93 (basolateral) side of the cell monolayers were maintained in an aerobic environment to
94 limit hypoxia-induced epithelial cell death. Using this system we investigated the
95 influence of different fermented juices (tomato and feijoa) as fermentable carriers of LP
96 DSM20205on the ability of the bacterium to improve *in vitro* intestinal barrier function
97 as measured by the trans-epithelial electrical resistance (TEER) assay. The survival of
98 LP DSM20205 in different fruit juices during *in vitro* digestion, in addition to its
99 adhesion capacity and potential cytotoxic effect on Caco-2 cells were also studied.

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100 ## MATERIALS AND METHODS

101 ### Bacterial strain and growth conditions

102 A lyophilized culture of *L. plantarum* DSM20205 (ATCC 8014) was obtained from
103 DSMZ (Germany), and was previously isolated from corn silage. The bacterial strain
104 was grown from frozen glycerol stocks according to the method described by Valero-
105 Cases and Frutos [16] on Man Rogosa and Sharpe (MRS) agar plates for 48 h at 37°C in
106 5% CO_2. Single colonies were then inoculated into 10 mL of MRS broth and grown 24
107 h at 37°C in 5% CO_2. This primary culture was inoculated into MRS broth at 1% (v/v)
108 and incubated for 24 h at 37°C in 5% CO_2 to obtain a biomass of approximately 10^8
109 colony-forming units per milliliter (CFU/mL). The cultures were harvested by
110 centrifugation at 2000 × *g* for 10 min at 4 °C, washed twice with sterile phosphate
111 buffer saline (PBS) and stored in glycerol at -80 ºC until use.

112 ### Fermented juices

113 Tomato and feijoa juices were manufactured by NZ Natural Juice Company (Napier,
114 New Zealand) and purchased from a supermarket in Palmerston North (New Zealand).
115 The ingredients in the feijoa juice were: water, feijoa pulp (27% p/v), sucrose, citric
116 acid (E330), xanthan gum as stabilizer (E415) and vitamin C. The nutritional
117 composition of the feijoa juice was as follows (g/100 mL): < 1 g of proteins; < 1 g of
118 total fat (0 g of saturated), 12 g of carbohydrates (sugars), 1 mg sodium and 30 mg
119 Vitamin C. The ingredients in the tomato juice were: tomato juice (98.5% v/v), lemon
120 juice (1% v/v) and sea salt (0.5% p/v). The nutritional composition of the tomato juice
121 was a follows (g/100 mL): 1 g of protein, < 1 g of total fat (0 g saturated), 4.9 g of
122 carbohydrates (sugars) < 1 g of dietary fibre, 105 mg of sodium and 225 mg of
123 potassium. The energy provided by the feijoa and tomato juices were 218 and 100
124 kJ/100 mL, respectively.

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125 The fruit juices were fermented with the bacterium by inoculating 0.5% (v/v) (10^6 CFU
126 /mL) LP DSM20205 into 50 mL of juice in sterile polypropylene screw capped tubes
127 and incubated at 37°C in 5% CO_2 for 24 h. Fermented juices were stored at 4°C until
128 use on the same day.

129 **Survival of *L. plantarum* DSM20205 in fermented juices during in vitro digestion**

130 The survival of LP DSM20205in fermented feijoa and tomato juices during *in vitro*
131 digestion was determined as described by Valero-Cases, et al. [17] Briefly, simulated
132 gastric juices were prepared in PBS acidified to pH 2.5 with 1M HCl (Sigma-Aldrich,
133 New Zealand) and 3 g/L of pepsin. Ten mL of each juice was added to the gastric
134 solution (40 mL) and incubated for 1 h at 37 °C in 5% CO_2. The reaction was stopped
135 by adjusting the pH to 7 with 1M $NaHCO_3$ (Sigma-Aldrich, New Zealand). Simulated
136 small intestinal juices were prepared by adding 4.5 g/L of bile salts and 1 g/L of
137 pancreatin (Sigma-Aldrich, New Zealand) and samples were incubated for 2 h at 37 ºC
138 in 5% CO_2.

139 **Microbiological analysis**

140 *L. plantarum* DSM20205 concentration was determined at three time points: after
141 fermentation, after simulated gastric digestion and after simulated small intestinal
142 digestion. Aliquots of each fermented juice were removed at each time point and serial
143 dilutions spread in triplicate on MRS agar plate and incubated for 48 h at 37 ºC in 5%
144 CO_2. The results were expressed as log CFU/mL for each sample.

145 **Cellular lines and growth conditions**

146 Stock cultures of the human intestinal Caco-2 cell line (ATCC HTB-37, Manassas, VA,
147 USA) were grown in T75 flasks as described by Anderson, et al.[18] Caco-2 cells were
148 cultured in Medium 199 (M199; GIBCO, Invitrogen Corporation) supplemented with
149 10% fetal bovine serum (FBS; GIBCO, Invitrogen Corporation, Auckland, NZ), 1%

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150 penicillin-streptomycin (PenStrep: 10000 units/mL penicillin G sodium salt and 10000
151 µg streptomycin sulphate in 0.85% saline; GIBCO, Invitrogen Corporation, Auckland,
152 NZ) and 1% non-essential amino acids (NEAA; MEM non-essential amino acids
153 solution; Sigma- Aldrich) at 37°C in 5% CO_2. The medium was replaced every 3–4
154 days and cells subcultured weekly at a ratio of 1:3. For all experiments Caco-2 cells
155 were used at a passage number between 30 and 35.

156 **Cytotoxicity assay**

157 To measure the Caco-2 cell viability and cytotoxicity, the stable water-soluble
158 tetrazolium salt assay (WST-1) was used, based on the reduction of tetrazolium salt to
159 formazan dye by cells with active mitochondria. Caco-2 cells were cultivated on 96 well
160 plates at a seeding density of 2 x 10^5 cells/mL for 48 h at 37 °C in 5% CO_2. The
161 treatments were: control medium (PBS), 8 log CFU/mL LP DSM20205 in control
162 medium, control tomato and feijoa juices, fermented tomato and feijoa juices, and
163 digested fermented tomato and feijoa juices. Serial dilutions (1/20 to 1/1280) were
164 prepared for each treatment in the M199 control medium supplemented with 1% non-
165 essential amino acids, from an initial dilution (1/10) prepared in PBS. After 48 h of cell
166 growth, the medium was removed from the wells of the plates, and treatments were
167 added in triplicate (100 µL/well) and incubated for 10 h at 37 °C in 5% CO_2. After
168 incubation, 10 µL of pre-warmed WST-1 reagent (Roche) was added to each well and
169 the cells incubated for 2 h at 37 °C in 5% CO_2. The absorbance (450-650 nm) of each
170 sample was immediately read in a spectrophotometer with a microplate reader
171 (FlexStation 3, Molecular Devices).

172 **Apical anaerobic co-culture model**

173 Caco-2 monolayers were grown on Transwell inserts (6.5 mm, polyester, 0.4 µm pore
174 size; Corning Incorporated) in M199 supplemented with 10% FBS, 1% NEAA and 1%

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175 PenStrep at 37°C in 5% CO_2 for 17-19 days until differentiated. Caco-2 monolayers
176 with an initial TEER more than 250 ohms/cm^2 were used for the assays. In previous
177 research it has been shown that the human intestine is in the range of 150-350
178 ohms/cm^2.[19] The initial Caco-2 monolayer resistance values were measured using an
179 electrode chamber (ENDOHM-12 tissue culture chamber; World Precision Instruments,
180 Sarasota, FL, USA) and voltohmmeter (EVOM Epithelial Tissue Voltohmmeter; World
181 Precision Instruments). The TEER was calculated by multiplying the resistance by the
182 membrane area (0.33 cm^2).
183 The day before the TEER assay, the apical medium was removed from Caco-2 cells
184 growing on Transwells. Cells were washed gently with PBS and 260 µL of M199 with
185 1% v/v NEAA added to each well. PenStrep was not added because it would limit the
186 viability of the bacterium, and conversely, FBS was not added because it would cause
187 bacterial overgrowth. The basal compartment medium was replaced with 810 µL of
188 fresh M199 with 10% FBS, 1% PenStrep and 1% NEEA.
189 On the day of the experiment the Transwells were transferred into the dual-environment
190 co-culture chamber in an anaerobic workstation using the process described by
191 Ulluwishewa, et al.[15] Three independent assays with three replicates per treatment (total
192 n = 9 per treatment) were carried out. After the baseline TEER reading was recorded,
193 the medium was replaced by the treatments where the apical aerobic cell culture
194 medium was removed from each insert and replaced with the following anaerobic
195 treatments: control medium, 8 log CFU/mL LP DSM20205 in control medium, control
196 tomato and feijoa juices, fermented tomato and feijoa juices, and digested fermented
197 tomato and feijoa juices. The basolateral chamber contained aerobic medium. TEER
198 was measured hourly for 19 h using a cellZscope controller and software
199 (nanoAnalytics, Germany).

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200 ***L. plantarum* DSM20205 adherence to Caco-2 epithelial cells**

201 After 20 h of the TEER assay, the adherence of LP DSM20205 to Caco-2 epithelial
202 cells in the apical anaerobic model was determined. Transwells (n = 3 per treatment)
203 were transferred to 24 well plates, the anaerobic medium of each insert was removed
204 and the Caco-2 monolayer carefully washed three times with PBS. Cells were lysed by
205 incubation with 1% Triton X-100 for 15 min at 37 ºC. The lysates were serially diluted
206 with PBS and spotted onto MRS agar. The plates were incubated at 37 ºC for 48 h. The
207 percentage (%) adhesion was calculated as % Adhesion = (V1/V0) x 100 where V0 was
208 the initial LP DSM20205count for each treatment before the TEER assay and V1 was
209 the number of viable LP DSM20205for each treatment following the TEER assay.

210 **Statistical analysis**

211 Results are expressed as the mean ± standard deviation. The mean comparison was
212 performed via SPSS v 21.0 software package (SPSS Inc., Chicago-Illinois-USA) using
213 a two-way analysis of variance (ANOVA) to analyse the WST-1 assay results and the
214 effects of *in vitro* digestions. Analysis of variance with repeated measures in analytical
215 data for TEER assay. If the effect was significant ($p < 0.05$), the means of individual
216 treatments were compared using the Tukey multiple range test. A log-transformation
217 was applied before analysis of the data sets obtained from the LP DSM20205growth
218 and viability during *in vitro* digestion results.

219 **RESULTS AND DISCUSSION**

220 ***L. plantarum* DSM20205 growth in tomato and feijoa juices**

221 **Figure 1** shows the concentration of live LP DSM20205 in tomato and feijoa juices
222 after 24 h fermentation at 37 ºC and after stimulated digestion. After fermentation, the
223 concentration of LP DSM20205 in the two juices was statistically different ($p < 0.05$).
224 For both juices, the viable cell concentration of LP DSM20205 increased during

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225 fermentation at 37 ºC with respect to the concentration of the inoculum (6 log CFU/mL).
226 However, LP DSM20205 grew better in tomato juice compared to feijoa juice (8.73 log
227 CFU/mL *vs* 6.78 log CFU/mL ($p < 0.05$)). The different cell concentrations in the
228 fermented juices could be related to the chemical differences between the juice matrices
229 such as lower initial pH in feijoa juice (2.9 *vs* 3.9 in tomato juice), lower pulp
230 concentration (27% *vs* 98.5% in tomato juice) and higher sucrose content compared to
231 tomato juice. In a previous study with blended carrot-orange juices at pH 4.9, the
232 growth of LP DSM20205 was from approximately 6 log CFU/mL to 9.13 log CFU/mL
233 during 24 h of fermentation, and monosaccharides and malic acid were the preferred
234 substrates for LP DSM20205, while sucrose and citric acid were not metabolized by LP
235 DSM20205 during 30 days of refrigerated storage[20]. In another study, Nualkaekul and
236 Charalampopoulos [21] investigated the influence of low pH and citric acid in different
237 fruit juices on *L. plantarum* NCIMB 8826 survival. In cranberry juice a rapid drop in
238 cell viability due to low pH (pH 2.5) was observed after one week of storage; while in
239 orange, blackcurrant and pineapple juices with a pH around 3.8, greater cell survival
240 was observed. However, the citric acid did not was metabolised by the bacterium and its
241 concentration remained unchanged during storage. Therefore, in line with these
242 published results, the growth of LP DSM20205 in the present study could be influenced
243 by the beverage composition. It seems that feijoa juice was a less favourable medium
244 for LP DSM20205 growth compared to tomato juice. In future research, an alternative
245 strategy to improve the concentration of LP DSM20205 could be to increase the
246 fermentation time to determine whether LP DSM20205 would be better adapted to
247 feijoa juice conditions. Despite differences in the growth of LP DSM20205 between
248 feijoa and tomato juices, our study showed that in both juices, the LP DSM20205

249 concentration was higher than the minimum recommended for a probiotic drink (10^6-
250 10^7 CFU/mL).[1]

251 ***L. plantarum* DSM20205 survival during *in vitro* digestion**

252 **Figure 1** shows the survival of LP DSM20205 during the different steps of *in vitro*
253 digestion. After 60 min in simulated gastric conditions (pH 2.5), the viable cell count in
254 both juices decreased compared to the initial concentration. However, LP DSM20205
255 survived better in tomato juice compared to feijoa juice (98.7 and 90.2% of survival
256 respectively). Although LP DSM20205 was less tolerant to low pH and the presence of
257 pepsin in digested feijoa juice, the decrease in concentration was less than one
258 logarithmic unit (from 6.82 to 6.15 log CFU/mL). Therefore, LP DSM20205 survived
259 well during gastric digestion in both juices.

260 After 2 h of digestion in conditions mimicking the small intestine, pH 7 and in the
261 presence of bile salts and pancreatin, LP DSM20205 remained relatively viable in both
262 juices (**Figure 1**). Nevertheless, LP DSM20205 had higher survival rates in tomato
263 juice (94.6%) during both steps of *in vitro* digestion compared to that of feijoa juice
264 (84.7%). Therefore, according to the results, the tomato juice matrix was the better
265 carrier for LP DSM20205 to reach the colon in higher concentration compared to the
266 feijoa juice (8.33 log CFU/mL *vs* 5.78 log CFU/mL, respectively). However, despite a
267 small decrease in LP DSM20205 viability, both juices were a good matrix to ensure a
268 high viability of LP DSM20205 after *in vitro* digestion. In previous studies with
269 blended carrot-orange juices and pomegranate juices and as carriers, LP DSM20205
270 also survived at high concentration (7 log CFU/mL and 6.71 log CFU/mL, respectively)
271 after the same *in vitro* digestion period. Moreover, when the blended carrot-orange juice
272 was supplemented with inulin, the LP DSM20205 survival after *in vitro* digestion was

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273 higher.[17, 20] Therefore, LP DSM20205 is able to survive the *in vitro* digestion period but
274 its final concentration can be influenced by the carrier matrix.

275 **Cytotoxicity assay**

276 To the best of our knowledge, this is the first study to use the WST-1 assay to
277 investigate the cytotoxicity of LP DSM20205 fermented fruit juices and digested
278 samples on Caco-2 cells (**Figure 2**). The results obtained with the WST-1 assay were
279 based on formazan absorbance values due to the reduction of tetrazolium salt by
280 mitochondrial dehydrogenases in active cells. In this assay, the higher the level of
281 formazan produced the greater the absorbance values will be, thus indicating a higher
282 number of viable cells and less cytotoxicity.[22] Cytotoxic effects were not observed at
283 any dilution following a 12 h exposure of Caco-2 cells to the different treatments
284 (metabolic activity greater than 70% compared to the control for all samples). The
285 fermented samples and digested fermented samples had the same effect on cell
286 metabolic activity as the unfermented samples (tomato and feijoa juices) and those with
287 LP DSM20205 in PBS ($p = 0.05$). Only the digested fermented feijoa juices showed
288 higher values than unfermented tomato juices in the 1:10 dilution ($p < 0.05$). The
289 similar values observed for different dilutions of the same sample suggest that LP
290 DSM20205 at the highest concentration (1:10) did not have any effect on Caco-2 cell
291 viability compared to the more diluted samples (1:1280). The presence of LP
292 DSM20205 in different fruit juices did not alter the Caco-2 cell metabolic activity
293 compared to that observed with the control juices. Overall, none of the treatments tested
294 had a biologically relevant effect on cell metabolic activity as it remained greater than
295 70% of that of the control medium group.

296 **Trans-epithelial resistance across Caco-2 cell layers**

297 **Figure 3** shows the effect of LP DSM20205 in different juices on the barrier integrity of
298 Caco-2 cells, as measured by the TEER assay in an apical anaerobic model. The drop in
299 TEER between 0 and 1 h displayed by all samples was expected because of the
300 disturbance of the Caco-2 cells during addition of the treatment following the initial
301 reading.[23] When the effect of LP DSM20205 in PBS was compared with PBS only
302 control (Fig. 3A), we observed that LP DSM20205 increased TEER between 7 h to 12 h
303 of incubation, reaching a maximum level at 10 h. However, after 13 h of co-culture the
304 TEER started to decrease, reaching lower values than control media. The fermented
305 and non-fermented juices also had different effects on TEER compared to the control.
306 The non-fermented tomato juices did not improve the TEER values when compared to
307 the control medium. However, the non-fermented feijoa juice did not alter TEER values
308 compared to control medium until 13 h of incubation. Instead, TEER values started to
309 decrease after 14 h, while TEER values in the control continuously increased until 19 h.
310 Nevertheless, the fermented tomato juices increased the TEER values in respect to the
311 control medium showing the highest increase (around 50%) from 7 h to the final
312 incubation time (19 h). In contrast, fermented feijoa juice had lower TEER values
313 compared to the control throughout the incubation period. A drop in TEER was only
314 observed for cells treated with digested fermented tomato juice and digested fermented
315 feijoa juice. This reduction was likely due to a detrimental interaction between the
316 intestinal enzymes and the Caco-2 cells resulting in the loss of barrier integrity. In
317 previous studies, the enzymes were treated before the TEER assay either by
318 denaturation in a water bath at 90 ºC for 5 min, followed by filtration through a 0.2 µm
319 membrane,[24] or removed by centrifugation and filtration.[25] However, in the present
320 study, to retain the viability of LP DSM20205, the enzymes could not be denatured,
321 centrifuged or filtrated. Therefore, our results suggest that the presence of bile salts

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322 and/or pancreatin was responsible for the loss of Caco-2 cell integrity. To the best of
323 our knowledge, this is the first time that digested juices fermented with LP DSM20205
324 have been used to investigate the effect on the integrity of intestinal Caco-2 cells. In
325 future studies it may be interesting to investigate the elimination of bile salts and
326 pancreatin from the digested fermented juices without impacting bacterial viability.
327 When the fruit juices were fermented with LP DSM20205, a notable influence was
328 observed on TEER values. The most potent effect was observed with fermented tomato
329 juice which increased TEER values indicating an increase in barrier integrity. These
330 effects may be due to the production of organic acids by the bacterium, which are
331 known to the influenced by the chemical characteristics of the substrate medium for
332 growth.[26] For example, a previous study reported that *L. plantarum* NCIMB 8826
333 produced short chain fatty acids (SCFAs) in cereal matrices.[27] Production of these
334 metabolites by lactic acid bacteria during fermentation support their positive effects on
335 TEER because SCFAs are known to directly stimulate epithelium growth and
336 function.[28] Additionally, it has been reported that high concentrations of malic and citric
337 acids are potential stimuli of intestinal inflammation.[25] The pH of the fermented juices
338 was neutralized to simulate conditions in the small intestine, however, the concentration
339 of citric acid in unfermented feijoa juice remained high. In a previous study, it was
340 demonstrated that malic and citric acid concentrations in cranberry juice caused the loss
341 of Caco-2 cell barrier integrity while the low pH did not affect TEER. However, when
342 the concentration of organic acids in the cranberry juice was reduced, a positive effect
343 on barrier integrity was demonstrated.[25]
344 Therefore, in the present study, TEER values were dependent on the fermented juice
345 carrier as compared to the control medium (PBS). This is a preliminary study and could

346 therefore be considered as a basis for future studies and to investigate specific
347 compounds in fermented tomato juice and feijoa and verify their effect on TEER.

348 **Adhesion to Caco-2 cell after TEER assay**

349 The ability of probiotic bacteria to adhere to intestinal cells is another parameter for
350 selecting probiotic action. The adhesion and invasion of pathogens in the intestine may
351 be inhibited by the adhered probiotic bacteria.[29-30] Adhesion of LP DSM20205in the
352 different fruit juices is shown in **Figure 4**. The results showed that LP DSM20205
353 remained viable in the apical anaerobic model with high levels of adherence in all
354 samples (>50%). However, significant differences were observed between samples
355 depending on the fruit juice. Although we have shown that digested fermented juices
356 did not improve barrier integrity in Caco-2 cells, the adhesion assay confirmed that in
357 digested samples LP DSM20205 had better adherence to Caco-2 cells. The results
358 showed higher percentages of adherence in digested fermented tomato juice than in the
359 digested fermented feijoa juice (77.8 and 61.9% respectively). LP DSM20205 in PBS
360 (control) and LP DSM20205in fermented tomato juice had the greatest adherence (83.5
361 and 85% respectively); while LP DSM20205adherence in fermented feijoa juice was the
362 lowest (52%). Saxami, et al. [30] showed that *L. pentosus* B281 and *L. plantarum* B282
363 grown in control medium (MRS broth) had high adherence to Caco-2 cells (60 and 65%
364 respectively). However, in a previous study when the adherence ability of probiotic
365 bacteria (*Bifidobacterium animalis* subsp. *lactis* BB-12, *Lactobacillus acidophilus* LA-5
366 and *Propionibacterium jensenii* 702) was investigated in different goat dairy foods
367 (yoghurt, stirred fruit yoghurts and ice cream), it was found that the carrier food affected
368 the adherence capacity. Fruit yoghurt was shown to improve the adherence capacity for
369 all the bacteria strains investigated.[5]

370 In conclusion, this study shows that the functional properties of *L. plantarum*
371 DSM20205 (growth, survival during *in vitro* digestion, adherence ability, epithelial
372 barrier integrity) are dependent on the type of fermented fruit juice. Although *L.*
373 *plantarum* DSM20205 in feijoa juice showed a high survival during *in vitro* digestion
374 conditions and greater adherence to Caco-2 cells compared to the undigested sample,
375 fermented tomato juice had the greatest effect on barrier integrity and adherence to
376 Caco-2 cells, together with the highest rate of survival after *in vitro* digestion.
377 Collectively, the results indicate that *L. plantarum* DSM20205 has potential to be used
378 as a probiotic in the production of fermented tomato and feijoa juices. Further work is
379 necessary to determine the influence of each fruit juice on probiotic properties to ensure
380 the delivery of probiotic bacteria in high concentrations for maximum health benefits.

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388 **ABBREVIATIONS**

CFU	Colony Forming Units
LP DSM20205	*Lactobacillus plantarum* DSM20205
MRS	Man Rogosa and Sharpe
PBS	phosphate buffer saline
SCFAs	short chain fatty acids
TEER	trans-epithelial electrical resistance
WST-1	water-soluble tetrazolium salt assay

389

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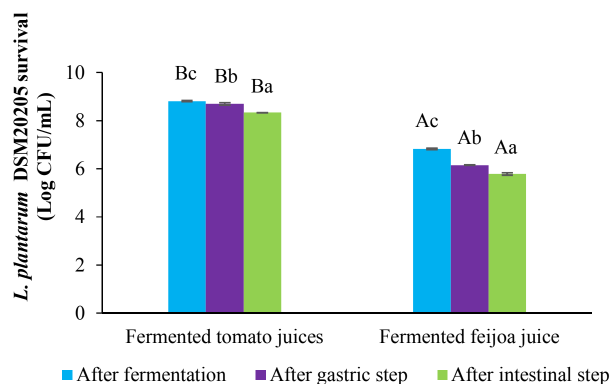

Figure 1. Effect of different fruit juices on *L.plantarum* DSM2005 growth and survival during different steps of *in vitro* digestion. The values represent the mean of 3 replicates and the error bars represent the standard deviation. Different lowercase letters denote significant difference on *L. plantarum* DSM2005 viability between different steps of *in vitro* digestion for the same type of juice. Different capital letters denote significant difference for *L. plantarum* DSM2005 viability between two juices for the same step of *in vitro* digestion ($p \leq 0.05$).

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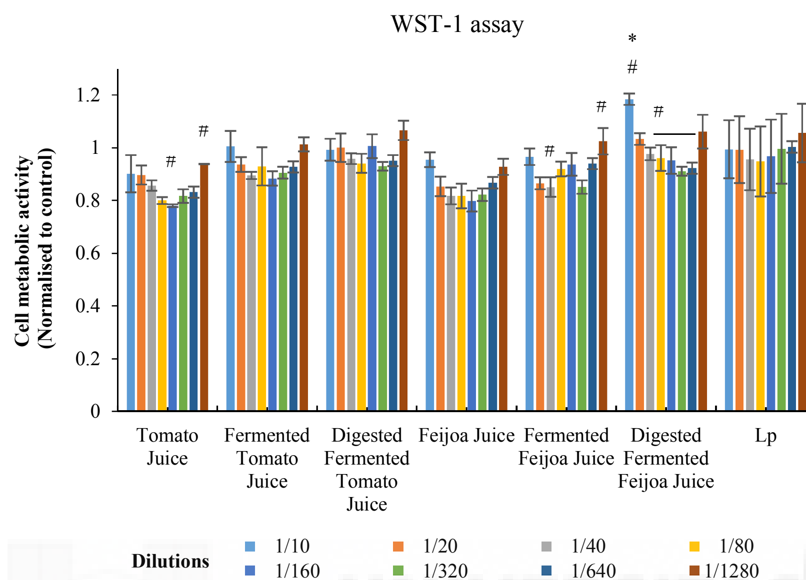

Figure 2. Effect of fruit food matrix and *L. plantarum* DSM20205 on the cell metabolic activity. Values are the mean of 3 replicates and the error bars represent the standard deviation. * denotes significant differences between different samples for the same dilution. # denotes significant difference between different dilutions for the same sample ($p \leq 0.05$)

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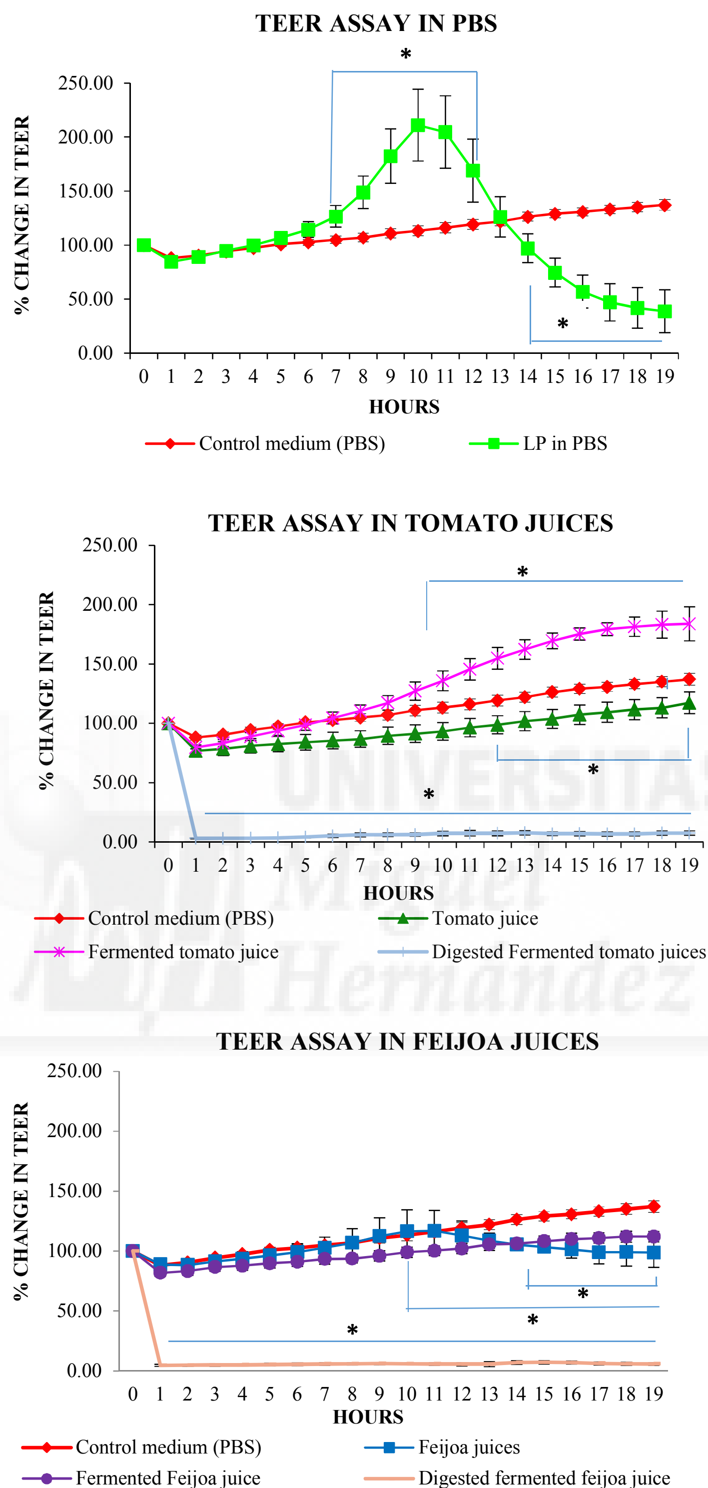


Figure 3. Effect of fruit matrix on the barrier integrity of Caco-2 cell monolayers cultured in the apical anaerobic co-culture model for19 hours. The values represent the mean of 9 replicates and the error bars represent the standard deviation. * $P < 0.05$ compared with control medium.

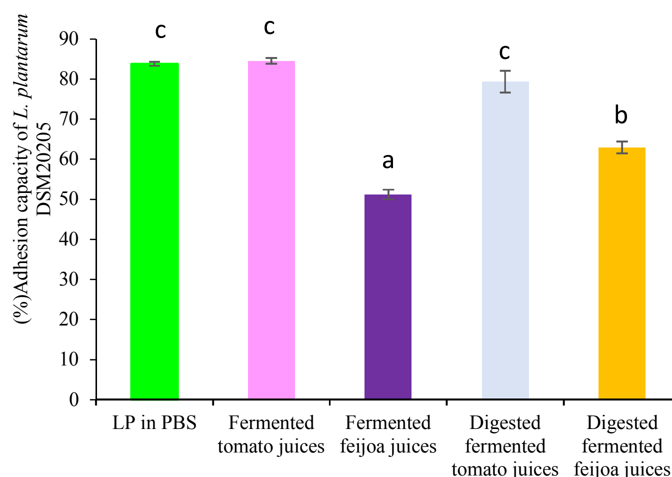
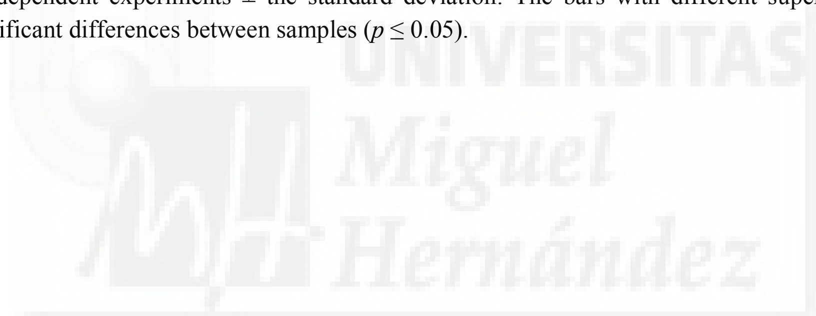


Figure 4. Effect of fruit matrix on adhesion capacity of *L. plantrum* DSM20205 to Caco-2 cells expressed as the percentage of probiotic bacteria adhered respect to the initial amount. The bars represent the mean of three independent experiments ± the standard deviation. The bars with different superscript letters denote significant differences between samples ($p \leq 0.05$).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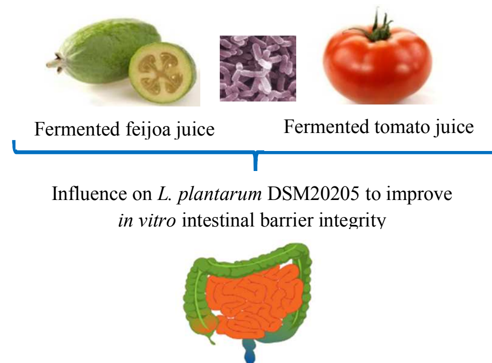

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RESULTS AND DISCUSSION

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4. RESULTS AND DISCUSSION

Currently, most of the published research on probiotics is focused on dairy matrices. However, there is a growing interest in developing new non-dairy matrices as probiotics carriers to offer consumers an alternative to fermented dairy products. Careful selection of the food matrix is an important factor to be considered in the development of probiotic foods, because the physicochemical and nutritional properties of the food matrices are crucial to ensure the probiotic viability. Therefore, the new alternative matrices must provide protection to probiotics during manufacture, storage and during gastrointestinal digestion, so that probiotics can reach the colon in high amount.

4.1. Effect of polymer matrices on the probiotic bacteria survival during manufacturing and storage

The first two manuscripts focused on the use of polymeric matrices (gelatin, agar-agar, carrageenan and alginate) to develop different gelled products and microcapsules (using different microencapsulation methods) with the aim of providing a high probiotic viability through the protective effect of the polymeric covering (patent ES2368401 B2 and LWT-Food Science and Technology 2015, 64:824-828, respectively).

Gelification in foods involves the association of polymer chains to form a three-dimensional network that traps water within it, forming a rigid structure. This structure can provide a good medium for probiotics, protecting them from the adverse environmental conditions and therefore ensuring their viability (Gaiani, 2011).

4.1.1. Gelled products

The first document is the patent “Probiotic or synbiotic gelled products and method for the production thereof” (ES2368401 B2), focused in the development of probiotic gelled products and their manufacture methods using different gelling agents. At present, a number of gelled products as jellies are in the market, but without live microorganisms in their formulations. This problem persists because of the difficulty of the probiotics for surviving during the manufacture process, because the gelling agent is dispersed in the liquid fraction, then mixed with the remaining ingredients and brought to

very high temperatures to induce the gelation process. To overcome this problem, the patented methods for the formation of gels implies the use of vegetable beverages as an insertion vehicle for microorganisms. Thus, a wide range of microbial concentrations can be achieved in the product formulation, representing considerable advantages with respect to the industrial usual procedures.

The basic method for obtaining gelled with probiotic products entails the following steps:

a) Sterilization and fermentation of the substrate.
b) Hydration of gelling agent (when necessary).
c) Dissolution of the gelling agent in hot water and bring to boiling temperature.
d) Aseptically cooling to approximately 40 °C.
f) Adding the fermented substrate and any other additives, homogenization of the mixture.
g) Packaging.
h) Refrigeration.

For the production of the probiotic gelled products, different polymers have been used, such as gelatine, carrageenan and agar-agar together with different percentages (15, 20, 30, 40 and 50 % v/v) of vegetable beverages such as tomato, carrot, peach and grape juices, soybean and rice drinks fermented with different bacteria (*Streptococcus salivarius* subsp. *thermophilus* CECT 986, *Lactobacillus delbruekii* subsp. *bulgaricus* CECT 4005, *Lactobacillus rhamnosus* CECT 288, *Lactobacillus casei* CECT 475, *Lactobacillus acidophilus* CECT 903, *Lactobacillus plantarum* CECT 220) alone or using various combinations of *St. thermophilus* and *Lactobacillus*.

In the development of a food product, it is essential to optimize the formulation in order to determine the optimum levels of the components (Dutcosky et al., 2006). In this experimental approach, the different factors considered to reach a high microbial viability were the type and amount of fermented beverage, the gelling agent and the probiotic microorganism. It was observed that in probiotic jellies (gelatin as gelling agent) with different *Lactobacillus* species alone or in combination with *St. thermophilus,* it was observed that the probiotic viability during storage (30 days at 4 ºC) depended on the

concentration of juice and on the type of microorganism. The best cell survival was achieved when 50% of the fermented beverages were added to develop the gelled products. The highest probiotic concentrations in jellies with 50% of juice were: 9.07 Log CFU/g (in carrot juices for *St. thermophilus* + *L. casei*), 9.04 Log CFU/g (in tomato juices for *L. acidophilus*) and 8.91 Log CFU/g (in peach juices for *St. thermophilus* + *L. delbrueckii* and *L. plantarum*). However, adding a 15% of each fermented beverage in the jellies, most of the microorganism concentrations were ≥8 Log CFU/g for in fermented juices during 30 days at 4 ºC. While jellies with tomato juice fermented by *St. thermophilus* + *L. casei* had the lowest viability (7.66 Log CFU/g). However, with only 15% of fermented beverage, the microorganisms survival was higher than the minimum recommended (10^6-10^7 CFU/mL or g) (FAO/WHO, 2002) during the storage. Moreover, the amount of fermented vegetable beverage to be added (15%) is within the range of juice that is currently used at industrial level in commercial gelled products.

On the other hand, inulin was also added for the development of synbiotic agar jellies, and it was observed an improvement in the microbial viability depending on the amount of inulin (0, 1 and 3 %). When 3% of inulin was added together 50% of tomato juice, *L. plantarum* reached the best survival (8.77 Log UFC/g) after 30 days of refrigerated storage Moreover, when lower concentrations of inulin (1%) and fermented juice (15%) were used the survival was still higher respect to the minimum recommended (≥ 8.17 Log CFU/g) after 30 days at 4 ºC. Therefore, the development of synbiotic gelled products could be also carried out using the lowest concentrations of fermented juices and inulin used in the assays.

The jellies formulated with carrageenan were developed using fermented soy and rice beverages (20 and 40%) as a vehicle insertion of *L. acidophilus* and *L. rhamnosus*, respectively. The results showed that although both microorganisms showed high survival levels after manufacture process (≥ 10^7 UFC/g), the carrageenan jellies elaborated with soy beverage fermented by *L. acidophilus* reached the highest cells concentrations (8.89 and 9.93 Log CFU/g with 20 and 40% of fermented beverage, respectively).

Therefore, it was observed that that the probiotic viability during storage depended more on the concentration of juice and on the type of microorganism than on the polymeric matrix used.

Another process included in the patent was the development of probiotic and synbiotic gummy candies and their preparation procedure using fermented vegetable beverages together with gelling agents. Commercial gummy candies are gel systems, basically made of sugars and gelling agents. These products are directed to a large and heterogeneous group of consumers: from children to elderly people (Cappa et al., 2015).

Therefore, their enrichment with fermented vegetable beverages would be a successful way to deliver probiotic microorganisms and at the same time sugar can be replaced by sweeteners. The fermented juices can be also incorporated into the candies directly during the normal manufacture process or through an injection system to act as filler. The results showed that the *L. plantarum* maintained high levels of viability during the manufacturing process and remained with concentrations of $\geq 10^8$ UFC/g during two months of refrigerated storage (4 ºC). However, further studies could be interesting to improve the commercial shelf life of the probiotic candies, for example using different packaging conditions to increase the probiotic microorganism viability.

4.1.2. Microcapsules

Another strategy to provide protection and improve the survival of the probiotic microorganism is through the microencapsulation (manuscript published in LWT-Food Science and Technology 2015, 64:824-828). The microencapsulation can be an alternative for introducing the microorganisms into the beverages or other foods without the need to ferment the final products. However, the vehicle for insertion of microorganisms into the microcapsules can be an important factor to improve the probiotic viability. A comparative study was carried out using different microencapsulation methods with alginate (extrusion and internal emulsion microencapsulation) and with the addition of inulin to investigate their protective and prebiotic effects respectively on the *Lactobacillus plantarum* CECT 220 (*L. plantarum*) viability during refrigerated storage. The protective effect of microencapsulation during the different steps of *in vitro* gastrointestinal digestion was also investigated. The *L.*

plantarum was added (live and active) directly to the Na-alginate solution at a concentration of 1:10 (microbial culture: Na-alginate solution). The results showed that depending on the methods of encapsulation and on the prebiotic content, there was a wide variation in bead size range. The size of the beads obtained by extrusion microencapsulation (EM) was higher (between 1.86 mm and 2.25 mm) and more homogeneous than that of the microcapsules obtained by internal microencapsulation (IM), with sizes ranging between 0.6 mm and 0.9 mm and with irregular shapes. The size of the microcapsules in the extrusion process could also be influenced by the alginate concentration, the diameter of the needle, the pressure on the syringe, the $CaCl_2$ concentration and the stirring speed in the solution where the alginate was dropped to form the capsules. Similarly, in the IM method, the size could be also influenced by the stirring speed during the encapsulation process, by the concentration of alginate or other compounds used for the encapsulation, and by the presence of insoluble particles of $CaCO_3$ suspended in the sodium alginate solution, that could interfere in the correct emulsion, leading to the production of microspheres with a wide size distribution (Cai et al., 2014).

At the same time, the different inulin concentration (1 and 2%) influenced both, the size of the microcapsules and the survival of *L. plantarum*. The capsules with 2% of inulin were significantly higher than those with 1% of inulin and without inulin, in both microencapsulation methods. However, the microcapsules with 2% of inulin, reached the highest survival values for *L. plantarum* in both types of microencapsulation at the end of the 30 days of storage at 4 ºC (6.66 Log CFU/g EMI2% and 6.61 Log CFU/g IMI2%). Therefore, the addition of 2% of inulin was used as a carbon source by *L. plantarum* after 15 days of storage period. However, the microbial survival in the capsules with 1% of inulin remained without significant differences ($p > 0.05$) respect to the other samples with or without inulin.

On the other hand, when the *in vitro* digestion was studied, it was observed that the different microencapsulation methods had also an influence on the *L. plantarum* protection through the *in vitro* digestion during the storage period. After 15 days of storage, the IM did not show a good protection trough the gastrointestinal tract and the *L. plantarum* was liberated in the gastrointestinal medium and subjected to higher environmental stress due to the changes in pH and to the interaction with the enzymes.

However, the encapsulation in EM was more resistant to gastrointestinal conditions and provided the best protection to *L. platarum* at the end of 30 days of storage, with a concentration of 7.40 Log CFU/g at the end of *in vitro* digestion process.

In general, when the polymeric matrices are used in gelled products and microcapsules to provide probiotic protection, evidence was found on the microbial protection during the manufacturing process and during a storage period of 30 days at 4 ºC, reaching high concentrations of probiotics in all gelled products (jellies, gummy candies) and microcapsules. However, for jellies and gummy candies, the probiotic viability was higher than for any of the types of microcapsules during storage. Moreover, the addition of 1% of inulin to the jellies was enough to improve the *L. plantarum* survival while with microencapsulation methods this inulin concentration was not enough to improve the microorganism viability.

The effectiveness of probiotic concentration in both encapsulation methods to achieve the same microbial viability as in the gelled products, could be improved by increasing the microcapsules number in the final products. However, this strategy could lead to an increase in the production costs and changes in the sensory perception. Muthukumarasamy et al. (2006), used capsules of the same size as those of the present study (2.1 mm) prepared by extrusion, to be included in sausages. The capsules were visible but were not detected by the panellists as they were similar to the fat particles of the product. Therefore, the size of the capsules and the type of food where they would be used, exert an influence in the sensory perception and thus in the consumer acceptance of the product.

Another alternative to improve the microbial concentration through the incorporation of microcapsules in food could be the use of fermented vegetable beverages as vehicle for the insertion of microorganisms into the capsules, following the same procedure as in the gelled products. However, in such beverages, as it has previously mentioned, various parameters, such as food matrix composition, prebiotics addition, type of microorganism, fermentation step and storage time, should be carefully controlled to ensure the probiotic viability at high concentrations and the microorganisms survival during gastrointestinal digestion process so that they can reach the colon in sufficient amount to confer the beneficial health effects. The impact of these parameters has been

also studied in different vegetable beverage matrices, to test their potential effect as carriers of different probiotic bacteria and this is part of the research of the following manuscripts:

- Carrot-orange juices fermented with *Lactobacillus plantarun* CECT 220 (*L. plantarum*). Plant Food for Human Nutrition (2017) DOI:10.1007/s11130-017-0601-x. From now on referred to as PFFN (2017).

- Carrot-orange juices and nectars fermented with *Lactobacillus acidophilus* CECT 903 (*L. acidophilus*). LWT-Food Science and Technology (2017, 81:136-143). From now on referred to as LWT (2017).

- Pomegranate juices fermented individually with one of the following bacteria: *Lactobacillus acidophilus* CECT 903 (*L. acidophilus*), *Lactobacillus plantarum* CECT 220 (*L. plantarum*), *Bifidobacterium longum* subsp. *infantis* CECT 4551 (*B. longum*), *Bifidobacterium bifidum* CECT 870 (*B. bifidum*). Journal of Agricultural and Food Chemistry (2017) DOI: 10.1021/acs.jafc.6b04854. From now on referred to as JAFC (2017a).

- Tomato juices and feijoa juices fermented with *Lactobacillus plantarum* DMS 20205 (corresponding to CECT 220) (*L. plantarum*). Journal of Agricultural and Food Chemistry (under review). From now on referred to as JAFC (2017b).

4.2. Effect of fermentation, prebiotics and beverage matrices on the probiotic bacteria growth

4.2.1. Prebiotic effect of inulin in juices and nectars on the probiotic bacteria growth

The carrot-orange juices and nectars were supplemented with different inulin concentrations (0, 1 and 3%) as prebiotic and fermented with *L. acidophilus* at 37 ºC during 36 hours (LWT, 2017). After fermentation time, comparing between the juices and nectars without inulin supplementation, the results showed that the juices without inulin (JIN0%) were a better substrate for *L. acidophilus* growth than the nectars without

inulin (NI0%), the concentrations of viable cells after fermentation were 8.34 and 8.03 Log CFU/mL in juices and nectars, respectively. However, in carrot-orange juices supplemented with different inulin concentrations (0, 1 and 2 %) and fermented with *L. plantarum* (PFFN, 2017), it was observed that after 24 h of fermentation at 37 °C, the growth of *L. plantarum* was about 9.13 Log CFU/mL in all juices. Although the fermentation time in carrot-orange juice with *L. acidophilus* was greater than carrot-orange juice with *L. plantarum* (36 *vs* 24h, respectively), *L. plantarum* growth was higher (9.13 Log CFU/mL) than that of *L. acidophilus* (8.34 Log CFU/mL). These differences in the bacterial growth would depend on the differences between the *Lactobacillus* species to adapt to the environment and on their ability to metabolize the substrate. At the same time, the differences in the initial pH between both juices (3.5 in juices fermented with *L. acidophilus vs* 4.9 in juices fermented with *L. plantarum*), may be the cause for a longer time for *L. acidophilus* to be adapted to the environment. Similar results were found when tomato and feijoa juices were fermented with the same strain of *L. plantarum* during 24 hours (JAFC, 2017b). *L. plantarum* grew better in tomato juice compared to feijoa juice, from an initial concentration of 6 Log CFU/mL to 8.73 Log CFU/mL and 6.78 Log CFU/mL, respectively. The different cell concentrations in the fermented juices could be related to the lower initial pH in feijoa juice (2.9 *vs* 3.9 in tomato juice) and also to the lower pulp concentration (27% *vs* 98.5% in tomato juice). Therefore, although in all beverages without inulin supplementation *L. acidophilus* and *L. plantarum* concentrations increased above $\geq 10^6$ CFU /mL, the type of beverage had an influence on the growth of *L. plantarum* and *L. acidophilus*. The *L. plantarum* achieved the best growth in carrot-orange juice followed by tomato juice and finally by feijoa juices. The *L. acidophilus*' growth was also influenced by the type of beverage (carrot-orange juices or nectars) achieving the best growth in orange-carrot juice.

Alternatively, the effect of inulin as prebiotic was studied after fermentation time in carrot-orange juices and nectars (manuscripts PFFN, 2017 and LWT, 2017). After 36 h of fermentation, it was observed that *L. acidophilus* reached the maximum viability (8.30 Log CFU/mL) in the carrot-orange nectars with the highest inulin supplementation (3%), at the same time, these nectars showed a decrease of 6 g/L of inulin. Nectars with 1% of inulin also had a positive effect on the microbial growth (although reaching lower concentrations than in nectars with 3% of inulin addition) on the *L. acidophilus* viability, reaching a concentration of 8.25 Log CFU/mL after fermentation time together with a

decrease of 2 g/L of inulin. Therefore, the inulin supplementation (1 and 3%) in carrot-orange nectars had a prebiotic effect on *L. acidophilus*. However, inulin supplementation in carrot-orange juices did not have any influence in the *L. acidophilus* viability after fermentation and monosaccharides were the only substrates metabolized by *L. acidophilus* during the fermentation period. Nevertheless, although *L. acidophilus* did not metabolize the inulin in juices during the fermentation period, its growth was still significantly higher ($p < 0.05$) than in the nectars.

The same results can be found in the study with carrot-orange juices with different inulin concentrations (0, 1 and 2 %) fermented with *L. plantarum*. As shown previously, the growth of *L. plantarum* was about 9.13 Log CFU/mL in all juices after 24 h of fermentation regardless of the inulin concentration. Therefore, the *L. plantarum* did not metabolize the inulin during fermentation in any sample, being glucose and fructose the main carbon and energy sources for this microorganism. In contrast, in the prebiotic carrot-orange nectars, inulin was the preferred substrate for *L. acidophilus* metabolism and was the main source of energy during fermentation. Nevertheless, when the inulin was not present, the *L. acidophilus* fermented the sucrose, with a higher decrease (3%) with respect to the synbiotic nectars resulting in a significant reduction in the total soluble solids (TSS) level.

The growth of *L. plantarum* and *L. acidophilus* in fermented vegetable beverages showed an increase in lactic acid along with a pH decrease. The extent of lactic acid production depended on the composition of the beverage and on the type of microorganism used for the fermentation of beverages, being higher in all fermented carrot-orange juices with *L. acidophilus* (around 2.5 g/L) than in all fermented carrot-orange juices with *L. plantarum* (around 2 g/L) with the lowest values in fermented carrot-orange nectars with *L. acidophilus* (around 1.6 g/L). At the same time, a decrease in malic acid was observed in all fermented beverages, with a higher loss in the fermented nectars (around 55% (w/v)) respect to the fermented juices (around 37% (w/v) with *L. acidophilus* and 30% (w/v) with LP). This indicates for *L. acidophilus* and *L. plantarum* the rapid transformation of monosaccharides and the conversion of the malic acid into lactic acid through the malolactic fermentation pathway carried out by these bacteria after the fermentation time (Garcerá et al., 2006). This implies that when the monosaccharides in the beverage are more limited, the probiotic can metabolize better the inulin as a

potential prebiotic source to improve its growth. Accordingly, it can be stated that the metabolism of inulin during the fermentation process was related mainly to the vegetable beverage matrix composition rather than to the probiotic specie or to the fermentation time.

4.2.2. Prebiotic effect of pomegranate juice polyphenols on the probiotic bacteria growth.

The potential effect of polyphenols on lactic acid bacteria (LAB) growth was studied in the manuscript JAFC (2017). In spite of pomegranate juices being a hostile ecosystem (pH, buffering capacity) and needing a long time for fermentation for the growth of LAB (Filannino et al., 2013), the concentration of all strains increased from 6 Log CFU/mL to 7.26-7.78 Log CFU/mL without significant differences ($p > 0.05$) among the probiotic bacteria used (*L. acidophilus*, *L. plantarum*, *B. longum*, *B. bifidum*). The microbial fermentation led to an increase in the levels of the phenolic compounds; a new catechin microbial derivative was identified after the fermentation with respect to the unfermented control juices. This microbial-derived catechin could be formed from the metabolism of other phenolic compounds (epicatechin and catechin) during bacterial fermentation, as it can be observed from the lower concentrations of these compounds in relation to the unfermented control samples. Epicatechin was almost completely metabolized (only traces were obtained in fermented juices) by *B. bifidum*, *L. plantarum*, and *L. acidophilus* and, to a lesser extent, by *B. longum* (3.59 mg/100 mL). Catechin was completely degraded by *B. bifidum* and *L. acidophilus* (only traces were obtained) and by *L. plantarum* and *B. longum* to a lesser extent (15.30 and 90.28 mg/100 mL were obtained, respectively).

On the other hand, the β- and α-punicalagin concentrations in the pomegranate juices fermented by *Lactobacillus* (*L. acidophilus* and *L. plantarum*) were lower than in the juices fermented by *Bifidobacterium* (*B. longum* and *B. bifidum*). The metabolism of ellagic acid derivative also depended of the different bacteria while the free ellagic concentration did not change during incubation time in any of juices. At the same time, it was observed that the antioxidant capacity was higher in the fermented samples for the three methods used (DPPH scavenging activity, ABTS scavenging activity and FRAP assays), respect to the unfermented ones, and the levels depended on the antioxidant

capacity method used and on the bacterial strain. Therefore, the LAB concentration increase during fermentation time could be in relation with the metabolism of most of the pomegranate phenolic compounds in a greater or lesser extent, depending on the probiotic bacteria strain.

In general, the results after the fermentation period in all fermented juices studied (carrot-orange juices, carrot-orange nectars, feijoa, tomato and pomegranate juices) showed the dependence of probiotic bacteria on the chemical characteristics of the substrate medium for growth; the latter is directly related to the bacterium's ability to produce organic acids from the carbon substrates (Bevilacqua et al., 2008). The inulin was only used as substrate during the fermentation time when the monosaccharides were limited as it occurs in the beverages prepared with the addition of water and sugar to juices (nectars). However, the pomegranate polyphenols were better metabolized during fermentation time in juices, indicating a possible prebiotic effect during that period. Nevertheless, in spite of differences in the growth of probiotic bacteria between the different vegetable beverage matrices, the previous results showed that in all juices independently of the presence of prebiotics (carrot-orange, carrot-orange nectar, tomato, feijoa and pomegranate juices), the probiotic bacteria concentration was higher than the minimum recommended for a probiotic drink (10^6-10^7 CFU/mL) (FAO/WHO, 2002).

4.3. Effect of storage time on the probiotic bacteria viability and beverage composition

4.3.1. Effect of inulin addition on beverage composition and probiotic bacteria viability during storage

The assessment of the physicochemical stability is needed to confirm that the fermented and non-fermented beverages remain with similar characteristics during storage maintaining at the same time suitable concentrations of probiotic bacteria in fermented beverages (Pimentel et al., 2015). Hence, inulin was used in different concentrations in both manuscripts PFFN, 2017 and LWT, 2017 with the aim of improving the stability of the chemical parameters and the microorganism viability.

Regarding the sugars content and inulin metabolism, in fermented carrot-orange juices with *L. plantarum*, it was observed that during the first eight days of storage period, only monosaccharides were metabolized by *L. plantarum*. However, between 10 and 15 days of refrigerated storage, in the fermented carrot-orange juices with 1 and 2 % of inulin supplementation (JIN1% and JIN2%, respectively), the inulin concentration decreased while the monosaccharides concentrations remained constant during this period. The fermentation of inulin may be favored by the decrease of nutrients after certain storage period (Grimound, 2010). Therefore, inulin consumption can be stimulated by the decrease of monosaccharides during the fermentation period and during the first eight days of storage. However, in the same storage period, the fermented juices without inulin (JIN0%) showed a decrease in the concentration of glucose and fructose as the only energy source. Nevertheless, in fermented carrot-orange juices and nectars with *L. acidophilus*, after 10 days of refrigerated storage, the inulin concentration did not change in any of the beverages during storage because the *L. acidophilus* metabolized monosaccharides and sucrose as the main energy sources in juices and nectars.

After 15 days, the viability of *L. plantarum* was higher for carrot-orange juices with inulin independently of the inulin concentration. Nevertheless, the survival of *L. plantarum* in JIN0% started to decrease progressively after 15 days of storage because of the lower concentration of monosaccharides at this stage. However, after 20 days of storage in fermented beverages with *L. acidophilus*, the highest survival of *L. acidophilus* (7.79 Log CFU/mL) was observed in the carrot-orange juices with 3% of inulin together with the highest inulin consumption. Nevertheless, in fermented carrot-orange nectars, an addition of 3% inulin was required to reach the same survival values (7.76 Log CFU/mL) as in the carrot-orange juices without inulin or with 1% of inulin. In this period, the monosaccharides concentration only decreased in the fermented juices and nectars without inulin supplementation (JIN0% and NI0%).

After 30 days, the *L. plantarum* population was higher in fermented carrot-orange juices with inulin (9.2 Log CFU/mL in JIN1% and JIN2% ($p > 0.05$)) than fermented carrot-orange juices without inulin addition (8.95 Log CFU/mL). Therefore, inulin improved the *L. plantarum* viability during the last 20 days of refrigerated storage, regardless of the inulin concentration (1 and 2%). At the same time, continuous inulin consumption by *L. plantarum* also lead to an increase in the monosaccharides

concentration in fermented juices (JIN1% and JIN2%) compared to fermented carrot-orange juices without inulin supplementation, where the total monosaccharides concentration in these juices (JIN0%) was 40 % lower with respect to the fermented juices with inulin. Therefore, the inulin addition in these beverages may be a good strategy for improving the stability of the monosaccharides during storage in these fermented beverages. Nevertheless, in all the fermented carrot-orange juices, the sucrose content did not change after the fermentation period and during the 30 days of refrigerated storage, indicating that sucrose is not metabolized by this strain of *L. plantarum*. On the other hand, at the end of the storage period (40 days), in fermented carrot-orange juices and nectars with *L. acidophilus*, the viability of *L. acidophilus* remained unchanged compared to juices and nectars without inulin, despite the sugars and inulin being metabolized by *L. acidophilus* in the synbiotic beverages during the last storage period.

Therefore, all beverages were a good medium to maintain the *L. acidophilus* and *L. plantarum* survival above the recommended concentration (10^6–10^7 CFU/mL or g) (FAO/WHO, 2002) during the storage period of 30 and 40 days, irrespective of the inulin concentration. However, only in fermented carrot-orange juices the inulin improved the *L. plantarum* survival during the last storage period.

Respect to the organic acid metabolism, in fermented carrot-orange juices with *L. plantarum* and *L. acidophilus*, the lactic acid concentration remained stable during the storage period. However, lactic acid concentrations in fermented nectars with *L. acidophilus,* increased throughout storage, although the levels remained lower than in fermented carrot-orange juices. Furthermore, it was observed that citric acid also decreased throughout the storage period when compared to the initial concentration but depending on the composition of the beverages. The fermented carrot-orange juices with *L. acidophilus* with and without inulin had a high citric acid decrease during storage (15.5 and 13.8%, respectively). However, in the fermented carrot-orange nectars with *L. acidophilus*, the citric acid decrease was lower but significant (around 4 %) during the storage period irrespective of the inulin concentration. Therefore, these results suggest that although citric acid was fermented by *L. acidophilus*, it did not become the main source of energy for *L. acidophilus* metabolism when compared to sugars and malic acid. However, in fermented carrot-orange juices with *L. plantarum*, the citric acid in all juices remained without significant changes ($p > 0.05$) (around 3.5 g/L) during fermentation and

storage meaning that it was not metabolized by *L. plantarum*. These changes in organic acid concentrations resulted in different pH and titratable acidity (TA) values during the storage period. The pH values decreased, and at the same time, in consonance with this decrease, the TA was shown to increase throughout the storage.

The results showed that the inulin addition in the different beverages improved their chemical composition, especially the sugars contents, probably having a positive impact on the sensory properties.

4.3.2. Hygienic quality in fermented beverages

To ensure the shelf life and adequate hygienic quality conditions it is necessary to perform a microbial evaluation throughout the storage period. This microbial evaluation was made in both manuscripts PFFN (2017) and LWT (2017), where the shelf life of carrot-orange juices and nectars fermented with *L. plantarum* and *L. acidophilus*, was studied. The microbiological analysis of moulds and yeasts showed that they were not detected during the assay in any carrot-orange juice fermented by *L. plantarum*. Nevertheless, the unfermented carrot-orange juice showed moulds and yeasts concentrations higher than >3 Log CFU/mL after 15 days of refrigerated storage. Therefore, the lactic acid contents in carrot-orange juices fermented by *L. plantarum* contributed to a lower pH and to the increase in shelf life, showing a good hygienic quality of the samples during the 30 days of refrigerated storage. However, in carrot-orange juices and nectars fermented by *L. acidophilus*, yeasts and molds were not detected in any sample throughout the 40 days of storage.

One difference between both studies was that carrot-orange juices were pasteurized prior to fermentation with *L. plantarum* (PFFN, 2017), while carrot and orange juices were sterilized prior to fermentation with *L. acidophilus* (LWT, 2017). Therefore, this indicates that the sterilization treatment of the carrot-orange juices and nectars was effective providing an adequate protection from contamination for at least 40 days at 4 ºC in all beverages. However, in pasteurized samples the hygienic quality and the shelf life were higher when the samples were fermented with *L. plantarum* after the heat treatment. Therefore, the lactic acid fermentation of the previously pasteurized

beverages is an interesting strategy to avoid microbial spoilage improving the beverages shelf life.

4.3.3. Sensory acceptability of the fermented beverages

The study of the sensory acceptance of the fermented beverages by the consumers during storage is interesting, because of the continuous physicochemical changes (decreases in sugars concentration and pH and increase in acidity) that may contribute to a negative acceptability by the consumers (Pimentel et al., 2015). The sensory acceptance was studied in the manuscripts LWT (2017). The sensory acceptance test of fermented carrot-orange nectars and juices with *L. acidophilus* supplemented with different concentrations of inulin were conducted the first day after the fermentation and during the storage (at 20 and 40 days). At the beginning of storage, there was no significant difference in the acceptability of the colour and aroma between samples. However, the beverage composition (juices or nectars) and inulin supplementation had an impact in the sweetness, acidity and flavour acceptance. Regarding the overall acceptance, the fermented nectars with inulin were the most accepted, while the unfermented control juices had the lowest acceptance. The fermented nectars supplemented with inulin scored the best in sweetness, acidity and flavour. It should be pointed out that acetic acid was not detected in any of the beverages. The absence of acetic acid after fermentation in the carrot-orange juices and nectars may result in a better acceptance, because, as previously described, this acid is related to distasteful flavor described as sour and vinegar (Salmerón et al., 2015).

During the storage period, the consumers assigned the lowest score in acidity acceptance to the fermented juices without inulin, probably because of the highest perception of the acidity due to lactic acid in the absence of inulin. However, the fermented nectars with the highest inulin addition (3%) were the samples with the best score for the acidity acceptance. These results suggested that the supplementation with 3% inulin along with sucrose helped to reduce the perception of acidity in nectars. Regarding the sweetness acceptance, taking into account that inulin has 35% of the sweetness of sucrose (Shoaib et al., 2016), the amount of inulin used in the supplementation of carrot-orange juices (1 and 3%) was not sufficient to contribute to the sweetness and reach the same acceptance values as in the nectars, where the presence of

sucrose together with inulin improved the sweetness acceptance. At the same time, the nectars fortified with inulin were the most preferred ($p < 0.05$) during storage (20 and 40 days), reaching the best score on the overall acceptance. Possibly the high acceptance values in sweetness and acidity influenced the overall acceptance score in these beverages. Therefore the substrate composition of fermented nectars with inulin addition had a positive influence on the sensory acceptance. While the other fermented beverages showed the same scores as the control unfermented samples. These results indicate that the changes produced by *L. acidophilus* on the chemical composition in the fermented beverages did not have a significant impact in the acceptance of the fermented samples when compared with the control samples.

4.4. Effect of *in vitro* digestion, prebiotics and beverage matrices on the probiotic bacteria survival

It is important to have food vegetable matrices that are able to protect the bacteria against hostile environments as the gastrointestinal tract. After evaluating the effect of fermentation, storage time and prebiotics on the composition of the beverages and on the growth and survival of probiotic bacteria, it is therefore interesting to further investigate the effect of *in vitro* gastrointestinal digestion of beverage matrices in the presence or absence of prebiotics, on the probiotics survival.

4.4.1. Carrot-orange juices with inulin as probiotic bacteria carriers under in vitro digestion during storage

The *L. plantarum* concentration in fermented juices after 30 days of storage was in the range of the recommended values (10^6-10^7 CFU/mL or g) (FAO/WHO, 2002), for reaching the colon in sufficient concentration after consumption. Therefore, it is also interesting to evaluate the effect of inulin on the survival of *L. plantarum* under simulated gastrointestinal digestion at different storage periods (1, 15 and 30 days) (PFFN, 2017). Comparing the *L. plantarum* survival in fermented carrot-orange juices with and without inulin supplementation under *in vitro* gastrointestinal digestion, the presence of inulin (1 and 2%) on the first day of storage had no influence on the survival of *L. plantarum* after gastrointestinal digestion, presenting values of 73% of survival in all fermented juices without significant differences ($p > 0.05$). However, after 15 days of storage, it was

observed that the inulin after *in vitro* digestion improved the *L. plantarum* survival with independence of the inulin concentration (1 and 2%). Nevertheless, at 30 days of storage, it was observed that *L. plantarum* survival was dependent on the inulin concentration after *in vitro* digestion, reaching percentages of gastrointestinal survival of 80, 75 and 73 % for fermented carrot-orange juices with 2, 1 and 0 % of inulin, respectively. Therefore, the results demonstrate that the presence of higher inulin concentrations could improve the survival of *L. plantarum* under intestinal conditions for long periods of storage. This could be due to the fact that the period of refrigerated storage and the intestinal conditions could limit the availability of sugars (Kimoto-Nira et al., 2010), favouring the consumption of inulin by *L. plantarum*. The beneficial effect of inulin on *L. plantarum* survival during the storage and gastrointestinal digestion is mainly observed after long periods of storage. In all samples, there was a high amount of viable cells after the gastrointestinal digestion (> 10^6 CFU/mL). Therefore, the carrot-orange juices without inulin are good matrices to ensure a high *L. plantarum* viability after *in vitro* digestion (6.62 Log CFU/mL), being the synbiotic juices with inulin the best carriers of *L. plantarum* due to the improved protective effect of inulin on the microorganisms, allowing them to reach the colon at higher concentrations (6.95 and 7.40 Log CFU/mL for concentrations of 1 and 2 % of inulin, respectively).

4.4.2. Tomato and feijoa juices as probiotic bacteria carriers under in vitro digestion

The survival of *L. plantarum* during the different steps of *in vitro* digestion was also studied in tomato and feijoa juices to verify their potential as carriers (JAFC, 2017b). After simulated gastric conditions (SGJ), *L. plantarum* survived better in tomato juice compared to feijoa juice as the microorganism was less tolerant to the low pH (98.7 and 90.2% of survival respectively). However, after simulated intestinal digestion (SIJ) *L. plantarum* remained still viable in both juices, being its survival higher in tomato juice (94.6%) than feijoa juice (84.7%) during both steps of *in vitro* digestion. The survival of the same *L. plantarum* strain (73%) in previous research in carrot-orange juices, was lower than in tomato and feijoa juices. However, in terms of concentration (CFU/mL) of *L. plantarum* that reached the colon, it was higher in tomato and carrot-orange juices (8.33 and 6.8 Log CFU/mL, respectively) than in feijoa juice (5.78 Log CFU/mL). The differences in *L. plantarum* survival during the *in vitro* digestion process could be

explained by the differences in substrate compositions. However, despite a decrease in *L. plantarum* viability after digestion, all juices were good matrices to ensure a high viability of *L. plantarum* after *in vitro* digestion in a high amount ($\geq 10^6$ CFU/mL).

4.4.3. *Pomegranate juice as probiotic bacteria carrier and biotransformation of phenolic compounds under in vitro digestion*

The bioaccessibility of microorganisms to some polyphenols is very low because of their degree of polymerization and glycosylation pattern (Etxeberria et al., 2013). Therefore, their physiological benefits depend on the quantity of phenolic compounds that are available (bioavailability) to be absorbed in the intestine (Chandrasekara & Shahidi, 2012; Fernandez-Garcia et al., 2009). The capacity of the lactic acid bacteria to metabolize the phenolic compounds depends on the species or on the strain (Cueva et al., 2010; Filannino et al., 2015). Although the health beneficial effects due to *Lactobacillus* and *Bifidobacterium* have been shown, their function as antioxidants has not been fully investigated (Mishra et al., 2015). Therefore, it interesting to study the effect of *in vitro* digestion on the metabolism and biotransformation of phenolic compounds by different species and strains of *Lactobacillus* and *Bifidobacterium* (JAFC, 2017a). In pomegranate juices, the simulated gastric juice (SGJ) conditions promoted the degradation of the epicatechin in the unfermented control juices with an increase (around 30%) in the catechin concentration in these juices after *in vitro* gastric digestion. With respect to the fermented pomegranate juices (FPJs), the survival of *B. longum* and *B. bifidum* (7.18 and 7.40 Log CFU/mL, respectively) in FPJs after *in vitro* gastric digestion was higher than in the juices fermented by *L. acidophilus* and *L. plantarum* (6.93 and 6.83 Log CFU/mL, respectively). These survival differences could be due to a higher metabolism of phenolic compounds by *B. longum* and *B. bifidum* during SGJ, with a higher decrease of α- and β-punicalagins and ellagic acid concentrations respect to the fermented pomegranate juices by *L. acidophilus* and *L. plantarum*. At the same time, a continuous increase in the antioxidant capacity was observed in fermented samples during the gastric digestion period, only for the DPPH and ABTS assays. This increase in antioxidant capacity could be due to the gastric conditions, with low pH and pepsin activity that led to an improvement in the release of bioactive antioxidant compounds such as epicatechin, catechin and α-punicalagin increasing their bioaccessibility, and thus their interaction with LAB.

However, after 60 min under simulated intestinal juices (SIJ1) an increase of ellagic acid in all fermented and unfermented pomegranate juices was observed, with amounts three times higher than those found after gastric digestion. The SIJ conditions (neutral pH, presence of pancreatic enzymes and bile salts) could promote the transformation of α- and β-punicalagins into ellagic acid (Larrosa et al., 2010). Consequently, a decrease was observed in those phenolic compounds (α- and β-punicalagins), with a higher loss (around 40%) of α-punicalagin content in the fermented juices with respect to the control unfermented juices (around 14%). This decrease could be related to the generation of a new α-punicalagin derivative that was only detected in fermented juices as a possible metabolite of the microbial transformations. The concentration in fermented juices with *L. plantarum*, *B. bifidum* and *L. acidophilus* were between 30 and 31 mg/100 mL, whereas the fermented juices with *B. longum* presented a lower concentration (25.07 mg/100 mL).

On the other hand, after SIJ1, decreases of catechin and epicatechin in the unfermented control juices were observed (around 45 and 64%, respectively). The decrease in unfermented control juices could be due to the instability of the catechin at neutral pH (Jilani et al., 2015; Krook & Hagerman, 2012; Marchese et al., 2014). In different studies with green and black tea, an important decrease was also observed in catechin after intestinal digestion (Jilani et al., 2015; Marchese et al., 2014). In this digestion step, these compounds (catechin and epicatechin) were not detected in fermented juices because of a previous biotransformation of these compounds due to de bacterial metabolism. The catechin derivative was only detected in fermented juices with respect to the control juices.

After 120 min of SIJ (SIJ2), following the same pattern as in SIJ1, the phenolic compounds metabolism observed in fermented juices was higher than in the control juices, probably because of the longer time of the fermented juices in intestinal juices with the microorganisms. The catechin microbial derivative (detected only in fermented juices) increased significantly in most of the fermented juices. In fermented juices with *L. plantarum*, *B. longum* and *L. acidophilus* this compound increased from trace amounts to 19.22, 6.47 and 9.45 mg/100 mL, respectively, whereas in the fermented juices with *B. bifidum* only traces of the catechin microbial derivative were detected. However, the α-

punicalagin microbial derivative concentration in SPJ1 was stable during this intestinal period. This fact may suggest that the bacterial metabolism of ellagitannins, catechin and epicatechin occurs during all the steps of the *in vitro* digestion, predominantly during the intestinal step. Generally, the metabolism in phenolic compounds for this period was higher for the fermented juices with *Bifidobacterium*. At the same time, it can be observed that the fermented pomegranate juices with *B. longum* presented the highest cell concentration at the end of the *in vitro* digestion. This fact could be related to the higher metabolism of most of the phenolic compounds in these fermented juices with *B. longum* with respect to the other fermented ones. However, the lowest cell survival was observed for the juices fermented with *L. plantarum* and the phenolic compound concentrations in these juices were higher than in the other FPJs. With regard to the cell concentrations, these results showed that although the LAB survival in SIJ2 was lower than in the other digestion steps, the microbial concentrations were still high (>10^6 CFU/mL) after all the *in vitro* digestion period.

At the end of the *in vitro* digestion, the antioxidant capacity for the DPPH and ABTS methods was higher in most of the fermented pomegranate juices respect to the control ones. Nevertheless, the FRAP assay values, showed a decrease in AOC for all juices after the all the gastrointestinal steps, in contrast to the DPPH and ABTS assays. This initial decrease in the fermented samples was significantly higher after 60 min under intestinal conditions. According to a previous study, the FRAP assay values of the control, fermented and digested juices under gastric conditions can be enhanced by the electron transfer reaction under acid pH conditions. The previously reported results for catechin instability at neutral pH in control juices and for the metabolism of the LAB in fermented juices, could be related to this decrease in FRAP antioxidant capacity after SIJ1 (Dejian Huang et al., 2005). A possible explanation is that catechin and epicatechin make an important contribution to the antioxidant activity of the juices due to their metal-chelating properties (Braicu et al., 2013). Therefore, the degradation of catechin and epicatechin in control juices and the formation of the catechin microbial metabolite through LAB metabolism in fermented juices after intestinal digestion could be associated with the reduction of the chelating activity, resulting in a lower antioxidant activity in the FRAP assay (Argy, 2006; Xia, 2010).

Therefore, the relationship observed in fermented juices between the phenolic compounds and cell concentrations suggests the possible prebiotic effect of phenolic compounds on the LAB. At the same time, the pomegranate juice was a good food matrix to ensure a high viability ($\geq 10^6$ CFU/mL) of all LAB used in this study after *in vitro* digestion, meaning that they can survive and reach the colon. The microbial metabolites derived from the fermentation of pomegranate juices and the high viability of the microorganisms in the colon may contribute to the maintenance of gut health. It is therefore interesting to make further research to test this hypothesis.

4.5. Effect of vegetable beverage matrices on the probiotic bacteria potential

Some aspects have been previously considered when evaluating bacteria as probiotics, such as their growth during fermentation time and their survival during manufacture or under gastrointestinal digestion, but there are other factors such as the efficacy of probiotic strains to adhere to intestinal epithelial cells and their ability to improve the intestinal barrier function, that should be investigated. Although the probiotic effect is known to be dependent on the bacteria strain, the functional properties of probiotics may change depending on the physicochemical properties of the carrier foods and ingredients used (Ranadheera et al., 2012). However, much of the research to evaluate the *in vitro* digestion tolerance and adhesion ability of probiotic bacteria has been carried out using dairy foods as the carrier matrix for the delivery of probiotics (Ouwehand at al., 2001; Ranadheera et al., 2012; Saxelin et al., 2010). Currently there is a lack of knowledge about the effect of fruit juices as carrier matrices on the activity and survival of probiotic bacteria (Martins et al., 2013). Therefore, the hypothesis for the research carried out was that fruit juices are a suitable carrier for probiotics and that they enhance the *in vitro* probiotic's ability to improve intestinal barrier function. To test this hypothesis, the influence of different fermented juices (tomato and feijoa) as fermentable carriers of *L. plantarum* DMS 20205 (corresponding to CECT 220) on the *in vitro* ability of this bacterium strain to improve *in vitro* intestinal barrier function, was investigated by the trans-epithelial electrical resistance (TEER) assay in an apical anaerobic model. In addition the *L. plantarum* adhesion capacity and potential cytotoxic effect on Caco-2 cells were also studied (JAFC, 2017b). To the best of our knowledge, this is the first time that digested juices fermented with *L. plantarum* have been used to investigate the cytotoxicity by WST-1 assay on Caco-2 cells and the effect on the integrity of intestinal

Caco-2 cells. The samples investigated were: PBS as control medium, *L. plantarum* in control medium (PBS), unfermented tomato and feijoa juices as control juices, fermented tomato and feijoa juices with *L. plantarum* and digested fermented tomato and feijoa juices with *L. plantarum*.

4.5.1. Cytotoxicity assay

To measure the Caco-2 cell viability and cytotoxicity, the stable water-soluble tetrazolium salt assay (WST-1) was used, based on the reduction of tetrazolium salt to formazan dye by cells with active mitochondria. Therefore, the higher the level of formazan produced the greater the absorbance values will be, thus indicating a higher number of viable cells and less cytotoxicity. The results showed that the fermented samples and digested fermented samples with *L. plantarum* had the same effect on cell metabolic activity as the unfermented samples (tomato and feijoa juices) and those with *L. plantarum* in control medium (PBS). Overall, none of the treatments tested had a biologically relevant effect on cell metabolic activity as it remained greater than 70% of that of the control medium group.

4.5.2. Trans-epithelial resistance across Caco-2 cell layers

The trans-epithelial electrical resistance (TEER) assay in an apical anaerobic model was used to measure the intestinal barrier function. Caco-2 cell monolayers (a human intestinal epithelial cell line) were grown in a novel dual-environment co-culture system that was used inside an anaerobic workstation (Ulluwishewa et al., 2015). The apical (luminal) side of the Caco-2 cell monolayers were maintained in an anaerobic environment to allow *L. plantarum* to have a similar metabolism to what would occur in the (anaerobic) colon. In contrast, the bottom (basolateral) side of the cell monolayers were maintained in an aerobic environment to limit hypoxia-induced epithelial cell death.

The results showed that the non-fermented tomato juices and non-fermented feijoa juices did not improve the TEER values compared to the control medium during all the incubation period. In contrast, a drop in TEER was only observed for cells treated with digested fermented tomato and feijoa juice. This reduction was likely due to a detrimental interaction between the intestinal enzymes and the Caco-2 cells resulting in

the loss of barrier integrity. In previous studies, the enzymes were treated before the TEER assay either by denaturing them in a water bath at 90 ºC for 5 min, followed by filtration through a 0.2 µm membrane (Yin et al., 2016), or removed by centrifugation and filtration (Serre et al., 2016). However, in the present study, to retain the viability of the *L. plantarum*, the enzymes could not be denatured, centrifuged or filtrated. Therefore, these results suggest that the presence of bile salts and/or pancreatin was responsible for the loss of Caco-2 cell integrity. In future studies it may be interesting to investigate the elimination of bile salts and pancreatin from the digested fermented juices without impacting bacterial viability.

Nevertheless, when the fruit juices were fermented with *L. plantarum*, a notable influence was observed on TEER values. Fermented feijoa juice had lower TEER values in respect to the control throughout the incubation period. However, the most potent effect was observed with fermented tomato juice which increased TEER values indicating an increase in barrier integrity (around 50%). These effects may be due to the production of organic acids by the bacteria, which are known to be influenced by the chemical characteristics of the substrate medium for the growth (Bevilacqua et al., 2008). For example, a previous study reported that *L. plantarum* NCIMB 8826 produced short chain fatty acids (SCFAs) in cereal matrices (Salmerón et al., 2009). The production of these metabolites by lactic acid bacteria during fermentation supports their positive effects on TEER because SCFAs are known to directly stimulate epithelium growth and function (Castro et al., 2016). Additionally, it has been reported that high concentration of malic and citric acids are potential stimuli of intestinal inflammation (Serre et al., 2016). The pH of the fermented juices was neutralized to simulate conditions in the small intestine. However, the concentration of citric acid in unfermented feijoa juice remained high. In a previous study, it was demonstrated that malic and citric acid concentrations in cranberry juice caused the loss of Caco-2 cell barrier integrity while the low pH did not affect TEER. However, when the concentration of those organic acids in the cranberry juice was reduced, a positive effect on barrier integrity was demonstrated (Serre et al., 2016). Therefore, in the present study, TEER values were dependent on the fermented juice carrier as compared to the control medium (PBS). This is a preliminary study and could therefore be considered as a basis for future studies and to investigate specific compounds in fermented tomato juice and feijoa and verify their effect on TEER.

4.5.3. *Adhesion to Caco-2 cells after TEER assay*

The ability of probiotic bacteria to adhere to intestinal cells is another parameter for testing the bacteria probiotic effect. The adhesion and invasion of pathogens in the intestine may be inhibited by the adhered probiotic bacteria (Messaoudi et al., 2012; Saxami et al., 2016). The adhesion capacity was also studied for *L. plantarum* in fermented and digested tomato and feijoa juices. The results showed that *L. plantarum* remained viable in the apical anaerobic model with high levels of adherence in all samples (>50%). However, significant differences were observed between samples depending on the fruit juice. Although it was previously shown that digested fermented juices did not improve barrier integrity in Caco-2 cells, the adhesion assay confirmed that *L. plantarum* in digested samples had better adherence to Caco-2 cells than the samples before *in vitro* digestion. The results showed that the percentages of adherence in digested fermented tomato juice were higher than in the digested fermented feijoa juice (77.8 and 61.9% respectively). However, the adherence of *L. plantarum* in control medium (PBS) and *L. plantarum* in non-digested fermented tomato juice was the highest (83.5 and 85% respectively), while *L. plantarum* adherence in non-digested fermented feijoa juice was the lowest (52%). Saxami et al. (2016) showed that *L. pentosus* B281 and *L. plantarum* B282 grown in control medium (MRS broth) had high adherence to Caco-2 cells (60 and 65% respectively). However, in a previous study when the adherence ability of probiotic bacteria (*Bifidobacterium animalis* subsp. *lactis* BB-12, *Lactobacillus acidophilus* LA-5 and *Propionibacterium jensenii* 702) was investigated in different goat dairy foods (yoghurt, stirred fruit yoghurts and ice cream), it was found that the carrier food affected the adherence capacity. Fruit yoghurt was shown to improve the adherence capacity for all the bacteria strains investigated (Ranadheera et al., 2012). Therefore, the *L. plantarum* ability to adhere to Caco-2 cells was strongly influenced by fermented juices as carriers showing the best adherence in fermented tomato juice.

CONCLUSIONS

5. Conclusions

1. The novel patented methods for the production of the probiotic and synbiotic gelled products are a new advance to offer an alternative to dairy probiotic foods.

2. The development of gelled products with probiotic concentrations above the minimum recommended (10^6-10^7 CFU/g) during manufacturing and storage, is feasible through the use of fermented vegetable beverages at low concentrations (15%) as an insertion carrier of probiotic bacteria.

3. The supplementation with the highest inulin concentration (2%) improves the *L. plantarum* CECT 220 viability during refrigerated storage regardless of the encapsulation methods. However, under *in vitro* gastrointestinal digestion conditions, the extrusion microencapsulation is the best method providing a higher protection and survival for *L. plantarum* CECT 220.

4. The lactic acid fermentation with *L. plantarum* CECT 220 increases the shelf life of the fermented carrot-orange juices respect to the unfermented ones. Moreover, the inulin addition (regardless the concentration) improves the *L. plantarum* viability and the stability of the juices composition during storage. However, the carrot-orange juices with the highest inulin content (2%) are the best matrices to ensure the delivery of *L. plantarum* at high concentrations in the large intestine after the *in vitro* digestion.

5. All fermented carrot-orange juices and nectars are good matrices to keep the *L. acidophilus* CECT 903 viability above the minimum recommended (10^6-10^7 CFU/mL) during 40 days of storage. Moreover, inulin improved the probiotic viability in carrot-orange nectars and their acceptance by the consumers.

6. Pomegranate juice is a good matrix to ensure a high microbial viability ($\geq 10^6$ CFU/mL) after the *in vitro* gastrointestinal digestion. The biotransformation of pomegranate juice phenolic compounds by *L. acidophilus* CECT 903, *L. plantarum* CECT 220, *B. longum* subsp. *infantis* CECT 4551 and *B. bifidum* CECT 870 has an influence on the antioxidant capacity and results in two new microbial phenolic

derivatives (catechin and α-punicalagin) suggesting a possible prebiotic effect of phenolic compounds.

7. The functional properties of *L. plantarum* CECT 220 (growth, survival during *in vitro* gastrointestinal digestion, adherence ability and epithelial barrier integrity) are dependent on the type of fermented fruit juice (tomato and feijoa), being the tomato juice the best matrix to improve the *in vitro* functional properties of *L. plantarum*.

8. The growth and survival of the probiotics during fermentation, storage and *in vitro* gastrointestinal digestion is influenced by the composition of the vegetal beverages used as potential carriers.

9. There are technological opportunities for the development of vegetal probiotic foods, using the different beverages and polymeric matrices as potential carriers for the microorganisms studied, in order to ensure their viability above the minimum recommended (10^6-10^7 CFU/mL or g) at the consumption time, to reach the large intestine in high amounts.

10. Further research is necessary to study through clinical trials, the physiological effects of the probiotic bacteria in the different fermented vegetable matrices.

5. Conclusiones

1. Los métodos novedosos patentados para la producción de productos gelificados probióticos y simbióticos son un nuevo avance para ofrecer una alternativa a los productos lácteos probióticos.

2. El desarrollo de productos gelificados con concentraciones de probióticos por encima de las mínimas recomendadas (10^6-10^7 UFC/g) durante el proceso de elaboración y almacenamiento, es factible adicionando bebidas vegetales fermentadas en bajas concentraciones (15%) como vehículo de inserción de bacterias probióticas.

3. La suplementación con la concentración de inulina más alta (2%) mejora la viabilidad de *L. plantarum* CECT 220 durante el almacenamiento refrigerado con independencia del método de microencapsulación. Sin embargo, bajo condiciones de digestión gastrointestinal *in vitro*, la microencapsulación por extrusión es el mejor método, proporcionando una mayor protección y supervivencia del *L. plantarum* CECT 220.

4. La fermentación ácido-láctica con *L. plantarum* CECT 220 incrementa la vida útil de los zumos de zanahoria y naranja respecto de los zumos sin fermentar. Además la adición de inulina (con independencia de la concentración) mejora la viabilidad de *L.plantarum* y la estabilidad de la composición de los zumos durante el almacenamiento. Sin embargo, los zumos de zanahoria y naranja con el mayor contenido en inulina (2%) son las mejores matrices para asegurar la liberación de *L. plantarum* en altas concentraciones en el intestino grueso tras la digestión *in vitro*.

5. Todos los zumos y néctares de zanahoria y naranja fermentados son buenas matrices para mantener la viabilidad de *L. acidophilus* CECT 903 por encima del mínimo recomendado (10^6-10^7 UFC/mL) durante 40 días de almacenamiento. Además, la inulina mejora la viabilidad probiótica en los néctares de zanahoria y naranja, así como su aceptación por los consumidores.

6. El zumo de granada es una buena matriz para asegurar una alta viabilidad de los microorganismos ($\geq 10^6$ CFU/mL) tras la digestión gastrointestinal *in vitro*. La biotransformación de los compuestos fenólicos del zumo de granada por *L. acidophilus* CECT 903, *L. plantarum* CECT 220, *B. longum* subsp. *infantis* CECT 4551 and *B. bifidum* CECT 870 tiene una influencia sobre la capacidad antioxidante y da lugar a dos nuevos derivados fenólicos (catechin and α-punicalagin) sugiriendo un posible efecto prebiótico de los compuestos fenólicos.

7. Las propiedades funcionales de *L. plantarum* CECT 220 (crecimiento, supervivencia durante la digestión gastrointestinal *in vitro*, capacidad de adherencia e integridad de la barrera del epitelio intestinal) dependen del tipo de zumo de fruta fermentado (tomate y feijoa), siendo el zumo de tomate la mejor matriz para mejorar las propiedades funcionales *in vitro* del *L. plantarum*.

8. El crecimiento y la supervivencia de los probióticos durante la fermentación, almacenamiento y digestión gastrointestinal *in vitro* se ven influenciados por la composición de las bebidas vegetales utilizadas como sustratos.

9. Existen oportunidades tecnológicas para el desarrollo de alimentos vegetales probioticos, usando las diferentes bebidas y matrices poliméricas como potenciales vehículos para los microorganismos estudiados, asegurando su viabilidad por encima del mínimo recomendado (10^6-10^7 UFC/mL o g) en el momento del consumo, para poder alcanzar el intestino grueso en elevadas concentraciones.

10. Es necesario seguir investigando mediante estudios clínicos los efectos fisiológicos de las bacterias probióticas en las diferentes matrices vegetales fermentadas.

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SCIENTIFIC PRODUCTION DURING THE PhD THESIS PERIOD

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7. SCIENTIFIC PRODUCTION DURING THE PhD THESIS PERIOD

During the period of the doctoral thesis, Estefanía Valero Cases has worked as an assistant professor in the Agro-Food Technology Department, Miguel Hernández University and in research projects ("*Evaluación de parámetros de calidad y seguridad de la producción agroalimentaria para su estimación mediante técnicas ópticas no destructivas durante su procesado y manipulación (ANA-DACSA*") and in the development of patent proof of concept ("*Gelatinas probióticas: desarrollo de producto y escalado industrial*").

DIRECTLY RELATED WITH THE PhD THESIS

Peer-reviewed papers

Valero-Cases, E., & Frutos, M. J. (2015). Effect of different types of encapsulation on the survival of *Lactobacillus plantarum* during storage with inulin and *in vitro* digestion. *LWT - Food Science and Technology, 64*(2), 824-828. doi: 10.1016/j.lwt.2015.06.049

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Valero-Cases, E., Nuncio-Jauregui, N., & Frutos, M. J. (2017). Influence of Fermentation with Different Lactic Acid Bacteria and in Vitro Digestion on the Biotransformation of Phenolic Compounds in Fermented Pomegranate Juices. *Journal Agricultural and Food Chemistry*. doi: 10.1021/acs.jafc.6b04854

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Relevant participation at international conferences

Valero-Cases, E., Frutos, M.J. (2016). Effect of different combinations of fermented pomegranate and grape juices with control juices on the physicochemical composition, microbial survival and consumer acceptance. EFFoST International Conference. 28-30 November, Vienna (Austria).

Valero-Cases, E., Frutos, M.J. (2016). Effect of fermentation with different lactic acid bacteria and *in vitro* digestion on the phenolic compounds of pomegranate juice. ICP International Conference on Polyphenols. 11-15 July, Vienna (Austria).

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Relevant participation at national conferences

Valero-Cases, E., Frutos, M.J. (2013). Nuevos alimentos probióticos elaborados con gelatina y bebidas vegetales. Congreso Nacional de Ciencia y Tecnología de los Alimentos. 12-14 Junio, Córdoba (España).

OTHER RELATED PUBLICATIONS AND CONFERENCE CONTRIBUTION

Peer-reviewed papers

Roda, A., De Faveri, D. M., Dordoni, R., Valero- Cases, E., Nuncio-Jauregui, P. N., Carbonell Barrachina, A. A., Lambri, M. (2016). Pineapple Wines Obtained from Saccharification of its Waste with Three Strains of Saccharomyces cerevisiae. *Journal of Food Processing and Preservation*. doi: 10.1111/jfpp.13111

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According to the Hippocrates´ aphorisms (460-370 BC) “Let food be your medicine and medicine be your food” and “All diseases begin in the gut”, nowadays, scientists studying the human microbiome suggest that healthy diets should include fermented foods to transiently strengthen living microbes in our gut. As a result, fermented food has gained popularity and consumers demand this type of food. However, most commercial probiotic foods in the market are dairy fermented foods and certain sectors of the population such as those allergic to milk proteins, strictly vegetarian and lactose intolerants, cannot consume them. Therefore, the need arises to explore new non-dairy matrices as carriers of probiotics to offer consumers an alternative to fermented dairy products. Accordingly, this PhD Thesis determine the influence of different vegetable matrices (polymeric matrices and beverages) as carriers of different potential probiotic bacteria in order to ensure their viability in the range of 10^6-10^7 CFU/mL or g of food at the consumption time, to reach the large intestine in high amounts.