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Study of the Stability of a Plant - Based Fermented Product and its Effect in a Murine Model of Type 2 Diabetes

Ph. D. Dissertation submitted by

Miriam Cabello Olmo

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Supervisors

Miguel Ángel Barajas Vélez, Ph.D.

Miriam Araña Ciordia, Ph.D.

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Dr. MIGUEL ÁNGEL BARAJAS VELEZ, Profesor Titular de la Universidad Pública de Navarra, y **Dra. MIRIAM ARAÑA CIORDIA**, Profesora Ayudante Doctor, ambos pertenecientes al Área de Bioquímica y Biología Molecular del Departamento de Ciencias de la Salud de la Universidad Pública de Navarra,

INFORMAN:

Que la presente Tesis Doctoral, **"Study of the Stability of a Plant-Based Fermented Product and its Effect in a Murine Model of Type II Diabetes"**, elaborada por **Dña. MIRIAM CABELLO OLMO,** ha sido realizada bajo su dirección, en la modalidad de Compendio de publicaciones, y que cumple las condiciones exigidas por la legislación vigente para optar al grado de Doctor Internacional y Doctor Industrial.

Y para que así conste, firma la presente en Pamplona, a 5 de Junio de 2020.

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"Knowing is not enough; we must apply.
Willing is not enough; we must do."
Johann Wolfgang von Goethe

"The important thing in science is not so much to obtain new facts
as to discover new ways of thinking about them."
William Lawrence Bragg

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A mis padres y mi hermana.

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Este trabajo es fruto de incuantificable trabajo, esfuerzo y dedicación, y sobre todo amor, mucho amor por la ciencia.

Declaration

The present doctoral thesis, entitled "Study of the Stability of a Plant-Based Fermented Product and its Effect in a Murine Model of Type 2 Diabetes" has been carried out by Ms. Miriam Cabello Olmo in the Department of Health Sciences of the Public University of Navarre (UPNA). This thesis is based on a compendium of three articles published in international peer-reviewed scientific journals, in co-authorship with the thesis supervisors, the thesis tutor, other members of the research group and collaborators from other groups.

The references of the papers are presented below:

I. Cabello-Olmo M, Oneca M, Torre P, Sainz N, Moreno-aliaga MJ, Guruceaga E. A Fermented Food Product Containing Lactic Acid Bacteria Protects ZDF Rats from the Development of Type 2 Diabetes. Nutrients. 2019;11(10):2530 (Impact Factor 4.171; Q1).

II. Cabello-Olmo M, Araña M, Radichev I, Smith P, Huarte E, Barajas M. New Insights into Immunotherapy Strategies for Treating Autoimmune Diabetes. Int J Mol Sci. 2019;20(19):1–26 (Impact Factor 4.183; Q2).

III. Cabello-Olmo M, Oneca M, Torre P, Díaz JV, Encio IJ, Barajas M, et al. Influence of Storage Temperature and Packaging on Bacteria and Yeast Viability in a Plant-Based. Foods. 2020;9(302):1–16 (Impact Factor 3.011; Q2).

These scientific papers are published under Open Access and are freely available in order to contribute with the scientific dissemination.

With the objective to fulfilling the stylistic requirements of a dissertation, the formats of the papers presented in the present thesis have been slightly modified and adjusted to the body of the manuscript. Such modifications did not affect the content of the publications.

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The current Ph. D. dissertation has been carried out in the Laboratory of Biochemistry of the Faculty of Health Sciences of the Public University of Navarre, within the framework of the project entitle "The role of synbiotics (Probisan®) in the prevention and development of diabetes and inflammatory bowel diseases in animal experimental models", granted from Advanced Innovation & Technology Corporation (ADItech Corp, Department of Economic Development, Navarre Government) [Reference: PT022 SIMDIACOL], and the study "Optimization of Probisan®'s manufacturing process for its future use in diabetic patients as a nutritional supplement" (Navarre Government) [Reference: 0011-1365-2017-000127], within the project HEALTHSTOCK (Ref. 733627) funded by the European Union Research and Innovation Programme Horizon 2020.

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I. One month stay in the Center for Research in Animal Health (Centre de Recerca en Sanitat Animal, CReSA), that belongs to the Institute of Agrifood Research and Technology (Instituto de Investigación y Tecnología Agroalimentaria, IRTA), located in the Autonomous University of Barcelona, in Bellaterra, Barcelona, Spain. (Aug. 2018).

II. Three months stay in Czech Republich in the University of Veterinary and Pharmaceutical Sciences Brno (Veterinárni a farmaceutická univerzita Brno, VFU) in Brno, the Central European Institute of Technology, (CEITEC) in Brno, and Agrobac (Agrobac s.r.o), a biotechnology company placed in Trnava, Zlin. (Sept. – Dec. 2018).

III. Three months stay in the Dairy and Food Science Department in South Dakota State University (SDSU), in Brookings, South Dakota, United States (Research stay co-founded by the Public University of Navarre) (Jul. – Oct. 2019).

List of Abbreviations

ADA	American Diabetes Association
BA	Biogenic amines
BB	Bio-breeding rat
BPB	Butyrate-producing bacteria
CLA	Conjugated linoleic acid
DM	Diabetes mellitus
EPS	Exopolysaccharides
FFs	Fermented foods
FMT	Faecal material transplants
FODMAP	Fermentable oligosaccharides, disaccharides, monosaccharaides and polyols
FPG	Fasting plasma glucose
GABA	γ-aminobutyric acid
GDM	Gestational diabetes mellitus
GM	Gastrointestinal microbiota
GRAS	Generally recognized as safe
GWAS	Genome wide association studies
HbA1C	Glycated haemoglobin A1C
HPA axis	Hypothalamic-pituitary-adrenal axis
IS	Immune system
IR	Insulin resistance
ISAPP	The International Scientific Association for Probiotics and Prebiotics
LAB	Lactic Acid Bacteria
LPS	Lipopolysaccharide
ME	Microencapsulation
MGWAS	Metagenome-wide association study
NOD	Non obese diabetic mouse
OGTT	Oral glucose tolerance test
RT-SP	Room temperature, standard packaging
SCFA	Short-chain fatty acid
T1D	Type 1 diabetes
T2D	Type 2 diabetes
TJs	Tight junctions
TMAO	Trimethylamine-N-oxide
WHO	World Health Organization
ZDF	Zucker diabetic fatty rat

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Summary

Probisan® is a registered fermented food product elaborated from a vegetable substrate and containing live microorganisms, including lactic acid bacteria and yeasts. Its main application is the supplementation of animal feed in livestock production. The present doctoral thesis investigates the potential effectiveness of Probisan® in the prevention and/or management of **type 2 diabetes mellitus**. In addition, the current dissertation also explores the effect of different **storage conditions** on Probisan®'s intrinsic characteristics, with the aim to refine its storage and maximize its functionality at the moment of consumption.

The growing incidence of type 2 diabetes and other non-communicable chronic diseases in the last decades has become a major concern for public health worldwide, since they are a leading cause of death, reduce life quality, cause social distress, and involve a substantial economic burden. In view of the ineffectiveness of current strategies to control diabetes, our microbial partners are presented as an alternative therapeutic target for the management of the disease.

The **gastrointestinal microbiota** performs a large number of functions, and imbalances in its activity could cause profound physiological changes and negatively affect host´s health. Regarding diabetes mellitus, scientific evidence indicates that there is an association between a dysfunctional microbiota and the diabetic disease. Furthermore, evidence-based knowledge suggests that certain dietary interventions may be appropriate to induce improvements in type 2 diabetes mediated by the gastrointestinal microbiota. In view of this situation, in this thesis we decided to evaluate the ability of Probisan® to restore normoglycemia and prevent and/or treat type 2 diabetes. The referenced product has previously demonstrated beneficial effects on livestock production, and therefore it is plausible that it could induce favorable effects in the clinical setting as well.

This thesis is based on three scientific publications: two research articles and one bibliographic review. Firstly, we tested the effect of Probisan® on the Zucker Diabetic Fatty rat, an experimental animal model of type 2 diabetes, for 31 weeks (***Study 1***). At the end of

the study we concluded that the administration of Probisan® in this animal model could not prevent the development of type 2 diabetes, however, it delayed the disease onset. In addition, we were able to confirm that Probisan® supplementation was favorable and alleviated complications and discomfort associated with diabetes, improving the health of the supplemented animals and increasing their life expectancy. We hypothesize that such protection was obtained through the modulation of the gastrointestinal microbiota. In any case, our study has certain limitations and more research is required to clarify this issue.

Secondly, we update the knowledge on the role of the gastrointestinal microbiota in the pathogenesis of **autoimmune type 1 diabetes**, another form of diabetes mellitus (***Study 2***). It is an extremely important topic since type 1 diabetes is an incurable disease and there is great heterogeneity in the response of patients to treatments, making it difficult to develop effective treatments for most patients. To do this, we conducted a narrative review of emerging therapies to treat the disease. In particular, we focus on those strategies based on immunotherapy, and in a specific section we delve into the role of the microbiota as a new approach to treat the disease. This study gave us a broader perspective to better understand the involvement of gut microbes in diabetes mellitus. Furthermore, it allowed us to explore possible therapeutic strategies to address type 1 diabetes through changes in the intestinal microbial ecology, both structurally and functionally.

Lastly, in order to better understand Probisan® and its behaviour over time, we aimed to evaluate the changes that take place in the product during its storage (***Study 3***). To analyse the physicochemical and microbiological properties of the product over time, we conducted a field study in which Probisan® was exposed to simulated storage conditions during its shelf life (1 year). For this, small sacks were prepared with Probisan® (150 g of product) that were stored in eight storage conditions [four storage temperatures (- 20 ºC, 4 ºC, and 37 ºC) and two types of packaging (normal packaging and vacuum packaging)]. The pH, moisture content, and counts of total bacteria, lactic bacteria, and yeast were determined at each sampling time (0, 1, 3, 6, and 12 months of storage). We were particularly interested in the study of microbial viability as we speculated that alive microorganisms could play a key role in the beneficial effects of Probisan®. At the end of

the study we were able to conclude that the microbial load of Probisan® is negatively affected in all the conditions studied, and this effect intensifies over time. Our results revealed that, in good agreement with our starting hypothesis, storage at low temperature (- 20 ºC and 4 ºC) protects better the viability of microorganisms in Probisan® compared to storage at room temperature (22 ºC) or high temperature (37 ºC). On the other hand, we did not find substantial differences between the packaging modes in the measured parameters, and vacuum packaging was ruled out as a possible strategy to preserve the load of alive microorganisms in Probisan®. Notwithstanding, alternative packaging approaches remain to be investigated.

On the whole, the observed results suggest that a dietary intervention with Probisan® could be useful for the clinical management of type 2 diabetes and probably other diseases, improving quality of life and wellbeing. However, considering the limitations of experimental animal studies, further studies are necessary to confirm the beneficial effects of Probisan®. On the other hand, although detailed research is essential to identify which Probisan®'s fraction(s) induce beneficial effects, the results of *Study 1* and *Study 3* suggest that the viability of microorganisms is not necessary to obtain health benefits. Therefore, future work should examine other potential health-promoting components of Probisan®, such as microbial metabolites generated during the production process, fermentable carbohydrates and other prebiotic compounds, and other bioactive molecules. Finally, this thesis aims to contribute to a better understanding of the functional properties of Probisan® and to propose future lines of research and applications.

Resumen

Probisan® es un producto alimenticio fermentado registrado elaborado sobre un sustrato vegetal y que contiene microrganismos vivos, entre ellos bacterias lácticas y levaduras. Su principal aplicación es la suplementación de la alimentación animal en la producción ganadera. La presente tesis doctoral investiga la potencial efectividad de Probisan® en la prevención y/o el manejo de la **diabetes mellitus tipo 2**. Por otro lado, esta investigación también explora el efecto de distintas **condiciones de almacenamiento** en las características intrínsecas de Probisan®, con el objetivo final de seleccionar las mejores condiciones para su almacenamiento y de este modo maximizar la funcionalidad del producto en el momento de consumo.

En las últimas décadas se ha observado una creciente incidencia de personas con diabetes tipo 2 y otras enfermedades crónicas no transmisibles. Dichas patologías se han convertido en uno de los principales problemas de salud pública a nivel mundial al ser una principal causa de muerte, reducir la calidad de vida, producir estrés social, y generar un sustancial gasto económico. En vista de la ineficacia de las actuales estrategias para controlar la diabetes, los microorganismos presentes en nuestra microbiota se postulan como una diana terapéutica alternativa para el manejo de la enfermedad.

La **microbiota intestinal** realiza un gran número de funciones beneficiosas para el hospedador, por tanto, desequilibrios en su actividad podrían causar profundas alteraciones fisiológicas y afectar de forma negativa a la salud. En lo que respecta a la diabetes mellitus, la evidencia científica indica que existe una asociación entre una microbiota disfuncional y la enfermedad diabética. Además, el conocimiento basado en la evidencia sugiere que ciertas intervenciones dietéticas podrían ser apropiadas para inducir mejoras en la diabetes tipo 2 mediadas por la microbiota gastrointestinal. En vista de esta situación, en la presente tesis decidimos evaluar la capacidad de Probisan® para restaurar la normoglucemia y prevenir y/o tratar la diabetes tipo 2. Este producto ya ha demostrado efectos beneficiosos en la producción ganadera, y por consiguiente, supusimos que podría inducir efectos beneficiosos también en el contexto clínico.

Esta tesis se basa en tres publicaciones científicas: dos artículos científicos originales y una revisión bibliográfica. En primer lugar, analizamos el efecto de la suplementación con Probisan® en la rata Zucker Diabetic Fatty, un modelo animal experimental de diabetes tipo 2, en un estudio que duró 31 semanas (***Estudio 1***). Al final del estudio observamos que la administración de Probisan® en este modelo animal no previno el desarrollo de diabetes tipo 2 aunque si retrasó el inicio de la enfermedad. Además de ello, pudimos confirmar que la suplementación con Probisan® fue favorable porque disminuyó las complicaciones y molestias derivadas de la diabetes, mejorando la salud de los animales suplementados e incrementando su esperanza de vida. Presuponemos que dicha protección se obtuvo mediante la modulación de la microbiota gastrointestinal. En cualquier caso, nuestro estudio presenta ciertas limitaciones y se requiere de más estudios para esclarecer el tema.

En segundo lugar, actualizamos el conocimiento sobre el papel de la microbiota gastrointestinal en la patogénesis de la **diabetes tipo 1**, otra forma de diabetes mellitus (***Estudio 2***). Se trata de un tema de suma importancia ya que la diabetes tipo 1 es una enfermedad incurable y existe una gran heterogeneidad en la respuesta de los pacientes frente a los tratamientos, lo que dificulta el desarrollo de tratamientos efectivos para la mayoría de enfermos. Para ello realizamos una revisión narrativa sobre las terapias emergentes para tratar la enfermedad. Particularmente, nos centramos en aquellas estrategias basadas en la inmunoterapia, y en una de las secciones del trabajo profundizamos sobre el papel de la microbiota como nuevo enfoque para tratar la enfermedad. Este estudio nos aportó una perspectiva más amplia para comprender mejor la implicación de los microorganismos intestinales en la diabetes mellitus. Además, nos permitió explorar las posibles estrategias terapéuticas para abordar la diabetes tipo 1 mediante cambios en la ecología microbiana intestinal, tanto a nivel estructural como funcional.

Por último, con el objetivo de conocer mejor Probisan® y su comportamiento en el tiempo, quisimos evaluar los cambios que tienen lugar en el producto durante su almacenamiento (***Estudio 3***). Para analizar las propiedades fisicoquímicas y microbiológicas del producto a lo largo del tiempo realizamos un estudio de campo en el cual Probisan® estuvo expuesto

a condiciones de almacenamiento simuladas durante su vida útil (1 año). Para ello se prepararon pequeños sacos con Probisan® (150 g de producto) que fueron almacenados en ocho condiciones diferentes [cuatro temperaturas de almacenamiento (-20 ºC, 4 ºC, 22 ºC and 37 ºC) y dos tipos de envasado (envasado normal y envasado al vacío)]. El pH, la humedad y los recuentos de bacterias totales, bacterias lácticas y levaduras fueron determinados en cada tiempo de muestreo (0, 1, 3, 6 y 12 meses de almacenamiento). Estábamos particularmente interesados en el estudio de la viabilidad microbiana ya que hipotetizamos que los microorganismos vivos podrían jugar un papel clave en los efectos beneficiosos de Probisan®. Al final del estudio pudimos concluir que la carga microbiana de Probisan® se ve afectada negativamente en todas las condiciones estudiadas, y que dicho efecto se intensifica con el tiempo. Nuestros resultados revelaron que, de acuerdo con nuestra hipótesis de partida, el almacenamiento a baja temperatura (-20 ºC y 4 ºC) protege mejor la viabilidad de los microorganismos en Probisan® en comparación con el almacenamiento a temperatura ambiente (22 ºC) o alta temperatura (37 ºC). Por otro lado, no encontramos diferencias importantes entre las dos formas de envasado en los parámetros de estudio, descartándose por tanto el envasado al vacío como posible estrategia para preservar la carga de microorganismos vivos en Probisan®. No obstante, futuros estudios podrían valorar otras formas alternativas de envasado.

En conclusión, los resultados observados insinúan que una intervención dietética con Probisan® podría ser útil para el manejo clínico de diabetes tipo 2 y probablemente otras enfermedades, pudiendo mejorar la calidad de vida y bienestar de los pacientes. No obstante, considerando las limitaciones de los estudios experimentales en animales, nuevos estudios son necesarios para confirmar los efectos beneficiosos de Probisan®. Por otro lado, aunque es imprescindible realizar una investigación detallada para identificar qué fracciones de Probisan® inducen los efectos beneficiosos, los resultados del *Estudio 1* y *Estudio 3* sugieren que la viabilidad de los microorganismos no es necesaria para obtener mejoras en la salud. Por consiguiente, futuros trabajos deberían examinar otros componentes presentes en Probisan® que pudieran generar un efecto beneficioso, como por ejemplo metabolitos microbianos producidos durante el proceso de producción, carbohidratos fermentables y otros compuestos prebióticos, y otras moléculas bioactivas.

Para finalizar, esta tesis pretende contribuir con un mejor entendimiento de las propiedades funcionales de Probisan® y proponer futuras líneas de investigación y aplicaciones.

Introduction

~ Chapter 1 ~

Fermented Foods and Food Microorganisms.

~ Chapter 2 ~

The Human Intestinal Microbiota.

~ Chapter 3 ~

The Role of the Intestinal Microbiota in Diabetes Mellitus.

~ Chapter 4 ~

Probisan®.

Chapter 1

Fermented Foods & Food Microorganisms

1. Fermented Foods

1.1. Introduction to Fermented Foods

The fermentation is defined as a metabolic process that implicates the microbial oxidation of carbohydrates until simple acids, and concurs with the production of organic acids (i.e., acetic acid, lactic acid), gas (carbon dioxide) and alcohol (ethanol), as well as a number of health-promoting components (i.e., bioactive peptides, antioxidants, antimicrobial metabolites such as reuterin and bacteriocins) (1,2). Since ancient times, fermented foods (FFs) such as cheese, fermented milk, sourdough bread, wine or beer have had a long tradition of acceptance and consumption in a large number of communities worldwide (3).

Concerning the role of FF in human health, Greek physician Hippocrates was one of the main promoters of the consumption of this type of foods, and broadly promulgated the beneficial effects of the FFs, particularly the alive microorganisms contained in them (4–6). Many centuries later, the awarded Nobel prize (1908) Russian microbiologist Elie Metchnikoff also insisted on the role of microorganisms in human's wellness (7). In the last years, FFs have been thoroughly investigated and today there is a vast array of newly acquired knowledge in the field, that encourages their consumption (8,9) and reinforces the putative role of microorganisms in health (10,11).

1.2. Classification of Fermented Foods

Currently there is a large number of FFs available on the marketplace that can be divided according to many different criteria. To start, a basic division would rely on the presence or absence of alive microorganisms. Similarly, they can also be classified on solid products or beverages. Another approach could be the historical use of FFs. On one hand, we find

traditionally manufactured FFs, like fermented milks (i.e., yogurt (12), kefir (13,14)) fermented tea (kombucha) (15), different forms of fermented soybean (i.e., chungkookjang, tamari, miso, tempeh, natto) (16–18), fermented cereals and grains (amasake (19), boza (20), idli (21)), fermented cabbage (sauerkraut, kimchi)(22), fermented fruit (yan-taozih (23), umeboshi (24)), pickled vegetables (horseradish, beets, cucumbers)(25) or fermented meat (sucuk)(3). On the other hand, there are innovative FFs formulated on unconventional matrices, such as peanut milk fermented with kefir culture (26) or coconut milk yogurt (25).

A typical and frequent pattern for the classification of FFs is the dairy or non-dairy origin. Dairy products account for the bulk of labelled FFs (27), nevertheless, given the changes in consumer's attitude towards dairy products (20,28), many non-dairy products are also available in the market (29,30). Lastly, another possible classification for FFs would be animal or plant based products, which would result similar to dairy and non-dairy criteria with the incorporation of meat and fish products along with dairy products. There are also some exceptions, like narezushi, that is the result of the combination of rice and fish fermented together for periods of up to months (31,32).

1.3. Significance of Fermentation in Foods

Fermented foods present a number of features and have a valuable technological and clinical interest. In general, as a result of the biochemical reactions that take place during the process, fermentation improves food's characteristics, provides with new chemical compounds and reduces undesired substances (15,33,34), as presented in **Figure 1, page 20.**

During fermentation, microorganisms produce *in situ* a variety of products with diverse properties (35,36). Generally, fermentation-derived products can be divided into two main categories: metabolites or microbial components. Regarding the former, many studies have demonstrated a rise in vitamins and minerals (i.e., vitamin A, B2, B11, B12, C, K, K2, selenium) (2,35,36), different enzymes (i.e., nattokinasa, maltase, invertase) (3,37,38) and

components with antioxidant properties such as tocopherol and phenolic compounds (1,17,29,39). Other desirable compounds produced during fermentation processes are conjugated linoleic acid (CLA) (40), γ-aminobutyric acid (41) or short-chain fatty acids (SCFA) (22). Further chemical compounds produced during the fermentative process are hydrogen peroxide, carbon dioxide, acetaldehyde and diacetyl (13,42,43). Last but not leasts, the bacteriocins are probably the most studied microbial-derived molecules. Bacteriocins, such as nisin, pediocin PA-1 and lacticin (44,45), are peptides or proteins with low molecular weight with a recognized bactericidal activity (1,40,46).

On the other hand, besides the aforementioned metabolites, a range of microbial structures and components with technological properties and beneficial effects (47) can split, be released into the food matrix and be ingested by the consumer (48). Amongst them, it stands out the exopolysaccharides (EPS) (i.e., kefiran, levan) (48–50), long-chain polysaccharides produced and secreted by a number of bacteria widely used in food industry (48,51) known to have many rheological properties (52,53) and induce health benefits (54–56). Other microbial components are cell-wall components like lipotechoic acids (57,58), techoic acids (40) and bacterial surface layer proteins (49,59).

Most of the aforementioned molecules and structures are often referred as **postbiotics**. They are defined as *"any factor resulting from the metabolic activity of a probiotic or any released molecule capable of conferring beneficial effects to the host in a direct or indirect way"* (60). This group of compounds have claimed beneficial effects (61), and in many cases induced effects comparable to those exerted by alive probiotic cells (62) including antimicrobial activity against potential pathogens (63), improvements in gut permeability or immunomodulation (64). Indeed, they can offer some advantages over alive probiotics like a greater stability during storage (62), a reduced risk of delivery of antibiotic-resistance genes (57) or less risk of developing adverse effects (57,62), what make them a potential alternative to probiotics for high-risk population groups (65,66).

It should be noted that during the process of fermentation, several modifications can occur, ranging from improvements in protein and lactose digestibility (28,38), greater bioavailability of certain nutrients (3,20,67,68), release of functional peptides otherwise

inaccessible for the hosts (36) to even lowering FODMAP components (fermentable oligosaccharides, disaccharides, monosaccharaides and polyols) and their associated digestive complications (39).

Fermentation can also mitigate some risks associated to the consumption of food by reducing the presence of some antinutritional factors such as phytate or trypsin inhibitors (38,69). In addition, fermentation can decrease levels of mycotoxins (2) produced by *Aspergillus, Penicillium* and *Fusarium* (70).

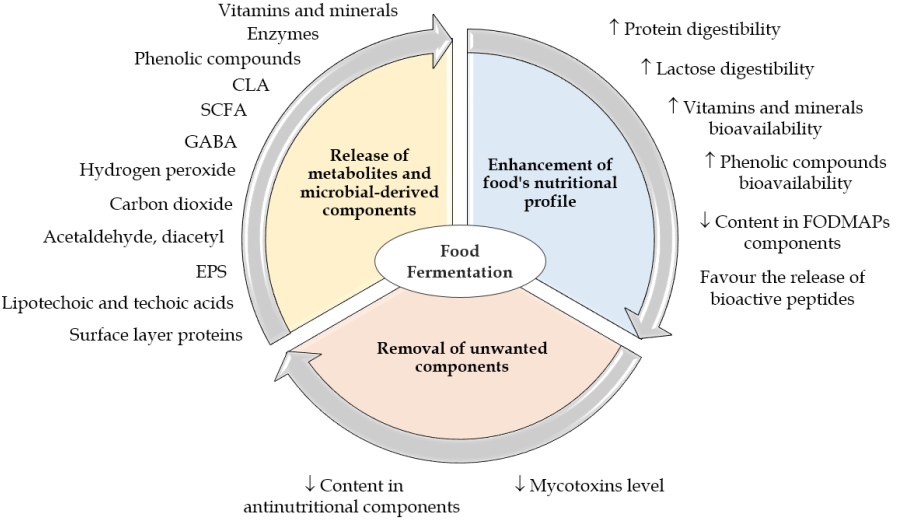


Figure 1. Summary of the main events occurring during food fermentation that provide fermented food with unique properties. CLA: conjugated linoleic acid; GABA: γ-aminobutyric acid; EPS: exopolysaccharides; FODMAPs: fermentable oligosaccharides, disaccharides, monosaccharaides and polyols; SCFA: short-chain fatty acids.

2. Characteristics of Fermented Foods

Fermented food refers to those foods and beverages that have been formulated through the cultivation of selected microorganisms in controlled conditions. As a consequence of substantial modifications occurring during fermentative reactions, FFs exhibit a number of attributes that differentiate them from the original raw materials (35). The sections below briefly address the main attributes of FFs (summarized in **Figure 2, page 24**).

2.1. High Stability

Typically, FFs are recognized as stable, and the existing literature confirms that fermentation and the incorporation of microorganisms in food can extend their shelf life (3,19,71). Moreover, fermentation can counteract the adverse effect of potential food pathogens due to the presence of substances with antibacterial activity (1), such as bacteriocins (38,72), enzymes (38), organic acids (73,74) and EPS (49). Similarly, alive microorganisms can also prevent the proliferation of food pathogens (2,75,76).

2.2. Unique Sensory Characteristics

Fermented foods present unique organoleptic properties. In general, they are very palatable foods, with strong flavours and present a special appearance (3,38). Some flavour-inducing metabolites that contribute to the organoleptic attributes are organic acids (73,77), amino acids (78), EPS (13) and acetaldehyde or acetoin (13,20,43). Similarly, microorganisms also participate in the organoleptic attributes of FFs (37). In addition to organoleptic changes, EPS and carbon dioxide can contribute to rheological properties (49,51) and food volume (3,35), respectively.

2.3. Health-Promoting Effects

Fermented products have proven to exert benefits on the consumer, and these properties are mainly due to the array of biogenic components they include. These products were introduced in section 1.2. and have proven to harbour immunomodulatory, antioxidant, antihypertensive, antiinflammatory, antiobesity and antidiabetic properties among others, as previously reviewed (29,39,40,79–82).

Furthermore, besides microbial metabolites and components, alive microorganisms present in many FFs are also a source of health-promoters (19,83). When they meet some specific criteria regarding safety, functionality and physiological and technological properties, these microorganisms are labelled as probiotics (38,43,84–87). **Probiotics** are defined as "*alive microorganisms that, when administered in adequate amounts, confer health benefits on the host*" (88). They exert numerous benefits on the consumer, most of them driven by the native microbiota (89,90), and their use in humans (61,91) and animals (92,93) has contributed with great health improvements. It is important to note that, although many FFs present probiotic microorganisms, this category of foods cannot be referred as probiotic (8). Besides the probiotic characteristic, FFs may also be a good source of prebiotic compounds. A **prebiotic** is defined as "*a substrate that is selectively utilized by host microorganisms conferring a health benefit*" (94), and are specially abundant in plant materials, specially cereals (19,30). In the past years, the term was used in essence for non-digestible fibers, however, the current definition also includes compounds of different origin such as polyphenols and polyunsaturated fatty acids (94). These characteristics, along with the array of bioactive compounds in FFs, make this type of food good modulators of the intestinal microbiota (34,95).

Regarding the beneficial effects reported for FFs, we found different healthy physiological effects beyond de nutritional role. Some recent reviews discussed the hypocholesterolemic, immunoregulatory, antitumoral, antiallergenic, antimicrobial, antioxidant, blood pressure lowering, antidiabetic, intestinal complication protective and body weight control effects (3,37,49,80,96,97). Furthermore, the consumption of certain FFs was associated to specific health outcome. To illustrate, improvements in diabetes-associated complications with

yogurt (97) and also with a specific plant-based fermented product (98), reduction in the risk for type 2 diabetes (T2D) with yogurt consumption (99), maintenance of bone health, antitumoral, antiinflammatory and antihyperlipidemic effects with soy-derived fermented products (17) and fermented milks (80), hypocholesterolemic effect in red yeast rice (100) and also antiaging effects in kimchi (101) have been reported.

2.4. Potential Risks

The consumption of FFs is not completely free of risk, and a number of health hazards have been associated with them. The main risks associated to FFs consumption are the presence of biogenic amines (BAs) and mycotoxins. The BAs, like histamine and tyramine, are small nitrogenous compounds generated as a result of an enzymatic reaction of amino acids decarboxylation (102). They are produced by a wide range or microorganisms (i.e., *Lactobacilli*, *Lactococci*, *Enterococci* or *Pediococci* genera) and are present in many food products of animal and plant origin (102–104). They are specially dangerous for groups with great sensibility (37) and produce a range of symptoms from headache and digestive difficulties to death (16,102). On the other hand, mycotoxins, such as aflatoxins, fumonisins and ochratoxins, are fungal secondary metabolites and can produce complications like haemorrhage, hepatotoxicity, neurotoxicity or immunosuppression (105,106). They particularly affect low income countries, especially African countries, where the manufacturing of FFs is frequently rudimentary, and the mismanagement of the crops or raw materials may favour contaminations with environmental microorganisms (106,107).

Lastly, other compounds derived from fermentative processes such as ethanol and acetaldehyde showed to be potentially detrimental for health, causing a leaky gut permeability and increasing the risk for alcoholic liver disease (108) and some types of cancer (106).

Overall, the available scientific evidence does not prove the danger of FFs and they could be considered as safe products in most cases (109).

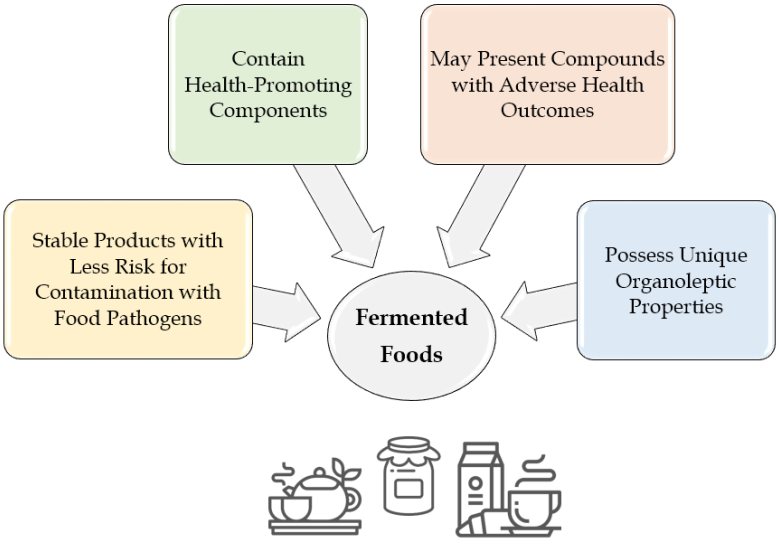


Figure 2. Summary of the main attributes of fermented foods.

3. The Importance of the Microbial Load in Fermented Foods and Probiotic Products

As described previously, the microorganisms present in FFs are an important source of health benefits. For this reason, food technologists and scientists aim to enhance the load of microorganisms and extend their viability over time during food storage. Some studies have revealed that some probiotic-containing food products present a load of probiotics considerably lower than expected (110). For that reason, it has become a central issue in foods containing alive microorganisms to guarantee the preservation of viable probiotics declared in the label until the opening time (111,112).

On the basis that the current definition of probiotic above referred entails the viability ("alive") and load ("adequate amount") (88), both requirements should be of great importance for probiotic food manufacturers. Indeed, bacteria viability during storage and

also after consumption is a very relevant issue for the properties of probiotic microorganisms (84). The term viability has attached different definitions over the course of the past years. Althought it was first defined as the ability of reproduce and have offspring, today's definition is slightly wider and more flexible. In a recent report, M. G. Wilkinson proposed a new conceptualization of "viability" that contemplates other relevant aspects such as metabolic, physiological and genetic functionalities of the cells (113).

Different loads of probiotic species have been explored in human trials (114,115) and in the development of functional food products (116–118). Althought the right amount of probiotic has not acquired a general consensus until now, it is speculated to range from 10^6 to $>10^{10}$ CFU per g/mL. Available data point to a probiotic effect with $>10^6$ CFU per g/mL, which is a widely accepted threshold for probiotic beneficial effects (43,119), however, most studies use a dose of 10^8-10^{10} CFU per g/mL of product (89).

In recent years, concerns have raised about the requisite of viability for probiotic cells's effectiveness, and some authors agree that bacteria viability is not a prerequisite for the obtention of beneficial clinical effects (40,57,62). At present there are many published works on disrupted probiotic or "paraprobiotics" (120) suggesting that they can induce effects comparable to those exerted by alive probiotic cells (62,65), can alleviate some health problems (57,121–123) and also be beneficial for other microorganisms present in the food product (47,48). On the other hand, others consider that despite non-viable probiotics can generate beneficial biological effects, the actions of viable probiotic cells are substancially more favourable for the host, and some outcomes seem to be restricted to alive probiotics (64). This depends on the mechanism underlying a given biological effect and the method used for the inactivation (57,64,124). Moreover, given the susceptibility of probiotics to storage, it is highly probable that evidence from clinical studies on probiotic species come from interventions in which at least a small fraction of the cells were dead at the moment of consumption (62,64).

4. Factors Affecting the Microbial Viability in Foods and Potential Strategies to Preserve Microbial Load

Considering the health properties of beneficial microorganisms present in functional foods (19,83,125), to promote the survival of bacteria, yeast and other beneficial microorganisms in food products is of special importance. The aim of this section is to review the major factors that influence microbial viability in food matrices. Particularly, I will focus on food-based products, whereas pharmaceutical forms such as dried powdered formulations will not be addressed profoundly. In the same line, I will not discuss those elements that interfere on the active site in the host after consumption.

The use of the term probiotic in the following paragraphs is not related to proven health benefits and is rather exclusively conceptual.

A few reviews have elegantly examined the main factors that regulate microbial viability in FFs and products containing probiotics. Briefly, authors insist on the importance of selecting the right probiotic strain, choosing a suitable food matrix, and controlling some factors relative to food processing, packaging and storage (43,84,126) (summarized in **Figure 3, page 32)**. Bacteria species present different sensibility to storage (85,118,127), temperature (128), pH (129,130), and osmotic pressure (131). For that reason, producers should select a suitable environment for a given probiotic microorganism taking into account food formulation, processing and storage.

4.1. Food Formulation

4.1.1. Food Matrix

Food ingredients and matrix strongly influence the viability of microorganisms (125,132) and at the same time they affect the functionality of microorganisms on the host (126). For that reason, it is important to study the combination of different probiotics and food matrices (133). Much research has been conducted on the topic, and the *Lactobacillus casei* T4 strain (134) along with *Lactobacillus plantarum* strains (135,136) seem to be good candidates for research in this area.

The incorporation of specific components during food production is an excellent technological approach for improving the production and storage of food products. Both microbial and non-microbial supplementary ingredients proved to be useful for avoiding some technical hurdle during food storage (137,138). To illustrate, the presence of protectants or antioxidants (43), prebiotics, fermentable sugars (136) and the content of nitrogen (139) may, to some extent, stabilize probiotic microorganisms and enhance their survival during food processing and storage. Some examples of growth promoters or protectants are, prebiotics and fibers, enzymes, micronutrients like folacin, whey proteinand other milk components (84,126), cysteine (140) or EPS from adjacent bacteria (40,47,48,52). On the other hand, the presence of antimicrobials or bacteriocins could compromise probiotics viability (84).

The porosity of the raw materials (135) and osmotic stress within the matrix (84) could also influence probiotics viability.

4.1.2. Chemical Characteristics

4.1.2.1. Acidity

pH value is apparently one of the most decisive factors on microbial viability (84,141) and metabolism (129), impacting on crucial activities like the synthesis of macromolecules and nutrients utilization (142). Most microorganisms grow well at neutral pH (pH of 7) but an extremely acidic environment is generally considered a growth-limiting factor (85,142,143) due to impairment of the enzymatic activity or cell membrane integrity (142).

The tolerance to acid varies widely among bacteria taxa (127,129,141,142,144). For instance, *Lactobacillus* spp. generally display a better tolerance to acidic conditions than *Bifidobacterium* spp. (130,131), and can establish and live in acidic environments such as the stomach or duodenum (145,146). Similarly, microbial sensibility to acidity seems to vary among food carriers and storage conditions (85,147) like storage time and temperature (148). The drop in pH in food products could be compensated thanks to the

buffering capacity of the substrate. To illustrate, the content in fermentable sugars during food formulation may attenuate bacteria sensitivity to acidic environments (136).

4.1.2.2. Moisture Content and Water Activity

The degree of hydratation is also of special interest in probiotic viability, specially in dried probiotics (137,149). Water activity is implicated in probiotic viability during storage (123) and low levels of water activity on food matrices seem to protect probiotic species (144). For this reason, the osmotic dehydratation may be a useful practice for reducing water activity and enhance probiotic viability over time (150,151).

4.1.2.3. Oxygen Content

The oxygen level, or dissolved oxygen present in a solution (152), is another stress factor that plays a major role in the loss of viability of probiotic species (126,143,153) owing to the fact that many of them are strictly anaerobic (42). Regarding LAB, not all LAB strains present the same sensibility to environmental oxygen (154), and physiological adaptations may happen (42).

The gas composition can limit the metabolism or promote the survival of some species of microorganisms, according to their sensibility to oxygen and others gases (155). Possible mechanisms for the destructive effect of oxygen are the peroxidation of cell components following bacteria exposure (43,152), or the accumulation of microbial by-products due to oxygen catalysis (42). Moreover, oxygen can also exert an indirect detrimental effect by impacting adjacent cells (85) or compromising other components such as phenolic compounds (134).

Regarding the strategies for reducing oxygen content, the incorporation of antioxidants or oxygen scavengers (i.e., ascorbic acids, catechins) is considered one of the best practices (43,156). Further approaches are vacuum packaging (118,157) or choosing packaging materials with low oxygen permeability (158), like glass containers (159,160). The use of microcapsules is another good approach for reducing the exposure to oxygen species (161).

In the same line, given the destructive effect of oxygen in most strains, in some cases it is advisable to perform the fermentation process under vacuum conditions (43) or reducing the redox level in the culture media (33).

4.1.3. Interactions within Microorganisms

Communication and cooperation between bacteria happens in nature, and is thought to be relevant for the beneficial effects of probiotic microorganisms as well (121,162). The nature of the probiotic microorganisms and their interplay with other probiotics or starter cultures is key for their survival during food processing and manufacturing (154). These interactions are especially important in multiple probiotic formulations (85) or complex mixture of microorganisms (i.e., starter cultures for kefir and kombucha) (13,34), where the presence of some microbes can possitively or negatively modify the final balance (155,160,163). To illustrate this cross-talk, metabolites from one bacteria, like bacteriocins, lactic acid, hydrogen peroxide and BAs, may be detrimental for others (27,73,85).

The selection of cooperative and no competitive species in multi-species fermentation processes is key in order to ensure that all the microorganisms have access to their nutrients (164). Moreover, bacteria resistance can also be induced with stress pretreatments, genetic modifications or by selective pressure (38,154,164).

4.2. Processing

Food processing entails significant viability losses and can affect the functionality of those alive microorganisms in food products (27). For that reason, all stages, including the selection of raw material, industrial processing and manufacturing, should be optimized with the objective to maintain the microbial load in food products (159,165). Given the degree to which industrial food processing can impact the survival of probiotic species,

many technological approaches can be addressed to enhance the quality of food as probiotic vehicles.

4.2.1. Fermentation Conditions

The conditions of the fermentation can deeply determine the physiological state of the bacteria (27). For that reason, the selection of the culture media (127) and the correct set of factors, like media pH (166) and incubation temperature (127), are key for bacteria culture due to their effect on growth kinetics. In the same way, the inoculation level (CFU/ml or portion) during food production also influences bacterial viability in the matrix (167).

The fermentation conditions can also affect the metabolism of bacteria and impact on the production of bacteriocins (129). Similarly, controlling the physiological state of the microorganism is of important consideration, as bacteria seem to be more resistant to external hazards in stationary phases in comparison to logarithmic phases (27,85).

4.2.2. Drying Process

Drying techniques are widely used for extending probiotics viability, and are useful for reducing costs associated to storage by avoiding the use of low temperature preservation (152). The main drying methods are spray-drying, vaccum-drying and freeze-drying (43,84). Besides its technological utility, drying process can cause high stress on bacteria and endanger their viability (38,135), and this impact varies according to the chosen method (168). For that reason, the drying method selection is a crucial aspect of probiotic food formulation. In addition, some strategies like the use of protectants, regulation of certain parameters (84) or physiological adaptations to extremely dry conditions (169), could mitigate the adverse effects of drying.

4.2.3. Microencapsulation

Given that the delivery mode is also important for the survival and the physiological effect of microorganisms in food products, a substantial body of research has focused on the use of the microencapsulation (ME) method for protecting microbial viability. Microcapsules behave like a shell that protects the cells from harsh conditions (36). For instance, they can increase bacteria tolerance to oxygen (154) and temperature (161,170) and can also mitigate the acidic gastrointestinal environment (171).

These microcapsules are composed of polymers of different materials that must be GRAS (Generally Recognized As Safe) certified materials (84) (i.e., starch, calcium-alginate, k-carrageenan, whey proteins, pectin, poly-l-lysine) (36,161). Among the system to envelop probiotics and create microcapsules, emulsion is probably the most extended one. However, other methods like spray drying, extrusion, phase separation, adhesion to starch and coacervation are frequently used as well (161,172,173). Both ME technique and material seem to influence the survival of microorganisms (84).

4.2.4. Packaging

Food packaging is another important factor to consider in food carrying probiotic microorganisms (174,175). As previously introduced, the modification of food packaging or materials is a good strategy to reduce the oxygen content in the food products. Some materials, like glass, have been reported to be less permeable and consequently maintain better bacteria viability (117,150). In other cases, however, the packaging material did not impact on the survival of probiotic species (176).

4.3. Storage Conditions

Storage conditions like temperature, relative humidity or light exposure, have been widely studied for their possible impact on microbial viability (43). Temperature can importantly affect the activity of microorganisms (144,177,178) and microbial sensibility to

temperature varies among bacteria strains (147). Refrigeration and low temperatures seem to preserve better the survival of probiotic species (162,179,180), however, exposure to sublethal aggressions can induce resistance and enhance the survival of some analysed bacteria taxa (181). Other factors like storage time (144,180,182) or exposure to light, specially in dried cultures (149), should be considered.

Environmental conditions can also impact on the bacterial metabolism resulting in the production of microbial compounds such as bacteriocins (129), and this phenomenon is of special interest for food production. Similarly, in some cases food storage and handling can influence oxygen permeability (84,175) or the presence of bioactive compounds in the food product, thus influencing probiotics indirectly (126). For instance, they can affect other components of the food matrix, such as antioxidants, and in that way affect bacteria viability (134).

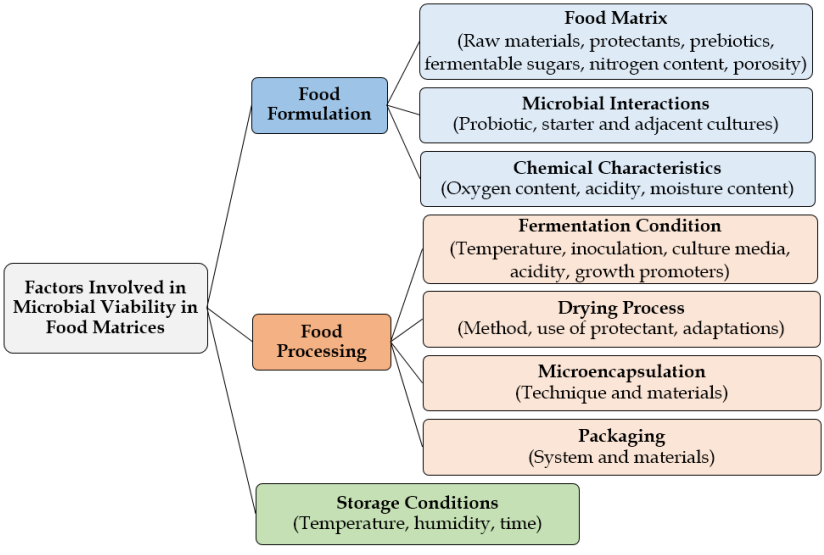


Figure 3. Summary of the main elements that influence on the microbial viability in fermented foods or probiotic-containing products.

Chapter 2

The Human Intestinal Microbiota

1. Introducing the Intestinal Microbiota

The human body is formed by a huge amount of microscopic creatures that coexist in a mutualistic relationship with the host's cells (183,184) They are predominantly anaerobic bacteria (185) but also viruses, yeasts, fungi, archaea and even protists (186). Lederberg and McCray first introduced the conception of **microbiota** in 2001, referring to it as "*the ecological community of commensal, symbiotic and pathogenic microorganisms that literally share our body space and have been all but ignored determinants of health and disease*" (187). These authors, nevertheless, were not pioneers in identifying the gut as a major determinant of health. The father of the modern medicine, Hippocrates, had already suggested that "*all diseases begin in the gut*" (188,189) and called for a focus on food as key determinant of health and disease (5,6,190).

The number of microorganisms in the human body overcomes the number of human cells, and it has traditionally been thought to represent 10 times the number of human cells (191). Recent reports with updated knowledge, however, indicate that a ratio of human cells to bacteria of 1:1 is more accurate (192). This microbiota is present along the whole body compartments including the skin, respiratory track, oral and vaginal cavities, but the vast majority is found in the gastrointestinal tract, named gastrointestinal microbiota (GM) (193). Specifically, the colon is by far the most colonized section, and its mass of microorganisms is thought to reach 1.5 kg of weight (194). It has been estimated that more than 100 trillion microorganisms inhabits this site (184), probably because of its intrinsic properties: oxygen-limited environment with access to many nutritive compounds from the diet, that makes it a suitable environment for several microbial forms of life. Besides exceeding in number the amount human cells, GM's genome, denominated *"microbiome"*, is also greater than humans' one, approximately 100 times greater (195).

The microorganisms that currently conform the mammalian GM have gone through a selection pressure and survived probably because of the advantageous role they play in the host wellness (196). An enormous diversity of bacteria have been found in the different body compartments (197) what may be explained to the divergent characteristics of each organ and tissue (146). The existence of site-enriched pathways in diverse parts of the body denotes that a familiarization period happened and allowed the resident microbes to adapt their metabolic functions to the location. More than that, one phylotype does not necessarily perform the same activities in different body sites, what highlights the metabolic adaptation degree (198).

2. Functions of the Gut Microbiota

Emerging evidence suggests that commensal bacteria and other less extensively studied unicellular organisms that inhabit the intestinal cavity have a strong influence on the well- functioning of the human body (82,185,199). This is because of the diverse array of activities they perform (reviewed in **Figure 4, page 39**).

Most of the knowledge concerning the functions of the GM has been generated, to a large degree, from research on germ-free animals (200). Several studies have indicated the clear influence of the microbiome in host physiology (201) and behaviour (202). Meaningful differences have been observed between germ-free and conventionally raised mice (203), not only in phenotypic features but also in important processes such as the metabolism and the digestive function (reviewed in (204)).

In order to explore the major functions of the autochthonous intestinal microbes, we should address their protective, metabolic and systemic functions.

2.1. Structural and Protective Role

The defence or protective role is probably the most relevant function GM conducts, being subject of increasing interest (183). The intestine is composed by two well-differentiated

parts, the small intestine and the large intestine. Both parts differ in size and width, and possess different histological characteristics (146). The small intestine's epithelium is the major responsible of the digestive process thanks to the pull of enzymatic substances that releases into the lumen (146), while the colon is home of the majority of the microorganisms that live in the intestinal track (146,183,205). Within the intestinal epithelium there is a complex system of protein structures, the apical junctional complex, that merges adjacent cells and acts as a gate regulating the exchange of molecules between the environment and the host (108). They are composed by the adherence junctions and the tight junctions (TJs). The TJs are formed by many proteins (broadly reviewed in (108)), and their permeability is governed by interconnected agents such as the diet (206), the structure of the microbiota (207), microbial-derived molecules such as SCFA (i.e., butyrate, acetate, propionate) (208) and also pathogens (108).

Besides TJs, the mucus layer that covers the epithelium is also vital for the maintenance of a well-functioning gut. Several functions have been attributed to the mucus, that works as a physical barrier between bacteria and host's cells (203,209) by regulating bacteria behaviour (210) and harbouring protective elements like anti-microbial molecules (211), immunoglobulin A (212) and immune cells (212). At the same time, the gut environment is highly affected by the composition of the GM, particularly butyrate-producing bacteria (BPB) such as *Roseburia* and *Faecalibacterium* genera, that promote mucin production and contribute to the development of a better barrier function and hence protection (213,214).

Since most of the immunological reactions take place in the intestinal mucosa (146), the epithelial cells from the intestinal mucosa have a relevant role in the regulation of tolerance and reactivity between somatic and microbial cells (215–217). The innate IS is the major responsible for the recognition of microorganisms via the pattern recognition receptos such as toll-like receptos and NOD-like receptors (186). The gut associated lymphoid tissue, that is a component of the mucosa-associated lymphoid tissue, is of pivotal importance for the symbiosis between the host and its GM. It is the largest lymphoid tissue in our body, and is responsible for the correct balance between our intestinal epithelium and the IS (218,219).

Considering the information presented above, the structural and the protective function of the gut microbiome are intimately connected. Overall, the permanent exposure to exogenous agents present in the lumen (including pathogenic microorganisms and dietary components among others) and the high number of immune cells in the intestinal track (mainly in the small bowel (146)), make the gut a relevant organ in the control of the host immune function (11,219,220).

2.2. Metabolic and Endocrine Function

The metabolic and fermentative potential of the GM has been well described, and such characteristics are partly attributed to the large amount of genes enclosed in the microbiome (203). Indeed, given the metabolic capacities of the GM, which possess a wide repertoire of endocrine functions (82), it is often referred as fermenter system or bioreactor (196,221). The structure of the GM will importantly limit its metabolic properties, as different proteolytic, lipolytic and saccharolytic profiles have been identified among human enterotypes, demostrating a differential fermentative capacity within bacteria taxa (222). The GM regulates many physiological processes in the host, and such control is mostly induced through chemical interactions by the exchange of molecules (203,223). Some molecules, like the autoinducers, are implicated in bacteria-bacteria communication (210), while others allow the dialogue between the host and their microbiota (223,224). The intestinal microbes also contribute with the synthesis of an array of metabolites and end-products (reviewed in **Table 1**). Further, some nutrients such as B12 vitamin are exclusively synthesize by bacteria (35).

The quoted microbial products are key messengers for the dialogue between the microbiome and the host, and are implicated in crucial functions. To illustrate, the GM influences energy harvesting from the diet regulating the utilization of monosaccharides from the digestion (195), the production of diet-derived microbial SCFAs (225) and the absorbed calories from the food (226). Similarly, it is implicated in food intake (controlling

satiety through gut peptides and adipokines (223)), glucose homeostasis and metabolism of glucose and lipids (82), and even the gastrointestinal transit (227).

Table 1. Compendium of gut microbiota-derived metabolites delivered to the host.

Metabolites	References
Amino acids (alanine, glutamic acid, lysine, valine)	(82,224,228,229)
Bile acids (cholate, hyocholate) and choline metabolites (methylamine, trimethylamine-N-oxide)	(82,186,203,224,228)
Hormones (cortisol, ghrelin, glucagon-like peptide-1, leptin)	(82,186)
Indole and indole derivates (serotonin, melatonin, 5- hydroxyindole)	(82,203,224,230)
Lipids (conjugated fatty acids, cholesterol, triglycerides)	(82,224)
Neurotransmitters (dopamine, serotonin, noradrenaline) and precursors to neuroactive compounds (tryptophan, L-dopa)	(82,186,228)
Phenolic and phenyl derivatives (benzoic acid, hippuric acid)	(203,224)
Polyamines (cadaverine, putrescine, spermidine)	(224)
Short-chain fatty acids (acetate, butyrate, propionate, valerate)	(82,186,224,228)
Toxins (TMAO, indoxyl sulfate, p-cresyl sulphate)	(186,203,230)
Vitamins (B2, B9, B12 and K vitamin, biotin)	(203,224,228)

Another important function is the transformation of nutrients from the diet into bioavailable compounds, prompting their biological function in the subject. This is of great importance for dietary polyphenols, that need enzymatic transformation to reach their biologically active form (aglycones) and then be available for the host (231). In this way, the intestinal microbes can impact on the efficiency of the digestive processes. In some cases the digestive role of the microbiota is disadvantageous, as some compounds can become toxic if metabolized by certain taxa of bacteria (reviewed in (232)).

On the whole, the metabolic function is intimately connected to the protective one. Gut-microbiota derived substances can importantly mediate in the host defence mechanisms through the regulation of the immune response, the inflammatory profile and also

controlling the gut permeability, for instance by improving the function of the TJs (203,210).

Besides the large number of products generated by the intestinal microbes, the host engages a bidirectional cross-walk with the GM, and can conversely regulate, to some degree, the characteristics of the GM by means of changes in pH, bile acids and some peptides and hormones (210).

2.3. Communication with the Brain and Central Functions

It is well known that some microbiome-generated substances such as serotonin, γ- aminobutyric acid (GABA), SCFAs, indole and secondary bile acids can exit from the gastrointestinal tract, migrate to the brain and behave like neurochemicals modulating the brain function and generating systemic effects through the microbiota-gut-brain axis (82,233). Conversely, the brain can also shape GM composition through the hypothalamic-pituitary-adrenal (HPA) axis (234).

Brain-GM communication is established through different mechanisms including neural, endocrine, immune and inflammatory signalling (233). This extended dialogue between the gut and the brain may explain the huge impact that the host circadian clock (235,236) and the different types of stress have on the intestinal tract (237,238) as well as the implication of the GM in the personality (239) and sleep quality (240). Further, an unbalanced GM has been linked to brain disorders like autism spectrum disorders (241), Alzheimer (242), depression or anxiety (233,243) and eating disorders (244).

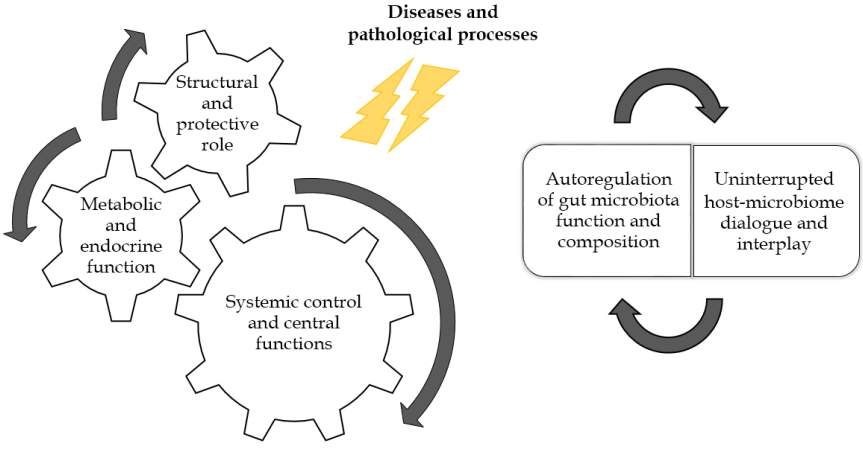


Figure 4. Integrative scheme of the regulatory functions performed by the gastrointestinal microbiota and its mutualism with host's physiology, whose disturbance result in disease states.

3. Implications of the Gut Microbiota for Health and Disease: a Double-Edged Sword

Given the diverse array of functions associated to the GM above described, it is reasonable that alterations on GM composition, activity or both, defined as *"**dysbiosis**"* (245), could lead to disease states and be the cause of profound disturbances in the host. A growing body of evidence suggests that the fitness of the GM dictates the risk for developing diseases, not only those associated to the intestinal track but also autoimmune disorders, metabolic diseases, mental health disorders and other conditions, as previously reviewed (listed in **Table 2**).

Humans have their first contact with the exogenous microorganisms at the time of birth, when they exit the utero and get in contact with the maternal vagina or skin depending on the type of delivery (246,247). From that moment, bacteria and other microbes start shaping the whole gastrointestinal system from the mouth to the anus. This process of

colonization lasts approximately three years (248) and is crucial for the right development of the commensal microbiota and the IS. It will in part determine the way our body interacts with microorganisms and the environment (249), for instance controlling the risk for autoimmune diseases, non-communicable diseases and gastrointestinal alterations (249–251).

It has long been known that a leaky gut with a dysregulation in TJs can alter the barrier function, allow the entrance of non-desirable molecules and subsequently cause a reactive response leading to pathological conditions, mainly gastrointestinal and inflammatory diseases (108). Epithelial cells and other structural components such as the mucus layer are of great interest for the right cross-talk between the host and its GM (252). An altered mucosa may led to important phenotypic features and it has already been associated with intestinal and extra-intestinal complications (108,200,216). Besides, structural and functional changes in the GM are strongly associated to profound changes on the host's physiology and hence diseases (82,218,220,253,254).

Table 2. Pathologies and conditions associated to the gut microbiome.

Diseases	References
Autoimmune diseases (Atopic dermatitis, celiac disease, eczema, multiple sclerosis, type 1 diabetes)	(108,216,220,227,242)
Cancer (Adenocarcinoma, colorectal cancer, ovarian cancer)	(200,218,220,242)
Cardiometabolic disorders (Atherosclerosis cardiovascular diseases, dyslipemia, metabolic syndrome, non-alcoholic fatty liver disease,	(82,200,218,227)
Degenerative disorders (Parkinson's Disease)	(82,200,242)
Diarrhea (Traveller's, antibiotic-associated)	(249,255)
Food allergies	(108,216)
Infections (*Clostridium difficile*, retrovirus, poliovirus)	(242)
Inflammatory bowel disease (Ulcerative colitis, Crohn's disease)	(108,200,218,220)
Irritable bowel syndrome	(82,108,218)
Mental disorders (Alzheimer's disease, anorexia nervosa, autism, depression)	(200,241,242,244,256)
Rheumatic diseases (Arthritis, ankylosing spondylitis)	(200,257)

Particularly, the microbial-derived molecules released by the GM have attracted a great deal of attention because of their role in the development or protection against diseases. They are often referred as postbiotics (60,61) and include structural components (lipopolysaccharide (LPS), peptidoglycan and flagellum) and metabolic products (reviewed above) (194,223). Many of them are associated with favourable health effects. For example, diet-derived SFCAs play a protective role in inflammatory processes because of their anti-inflammatory properties (82,203,237,258). Moreover, SCFAs have a pivotal role in host's metabolism because they are nutrients for colonocytes (259), participate in host´s metabolism of fatty acids, cholesterol and glucose (258), or promote the release of anorexigenic peptides (224). On the other hand, other microbial materials can be harmful and induce a diseased state. To illustrate, many research works have proven the harmful effect for the bacterial lipopolysaccharide (LPS). LPS is a cell wall component in gram negative bacteria that causes metabolic endotoxemia when enters the circulatory system (260). It induces an inflammatory response (261–263) that underlies insulin resistance (264) and obesity (221,262). Another microbial-derived metabolite that can treat health is trimethylamine-N-oxide (TMAO) produced in the transformation of choline and considered a potent predictor of cardiovascular disease (224,265), mainly because of its involvement in the atherogenic process (194).

In the same line, disruptions in the diversity of microorganisms in a given microbiota, measured with ecological parameters such as alfa and beta diversity or shifts in the relative abundance of certain groups, also correlate with host's fitness (184). Considering that not all microbial members display the same metabolic pathways, the friendly or unfriendly metabolome of the GM is tightly linked to the identity of its microorganisms (223). In this way, the presence of certain bacteria, such as mucin-degrading *Akkermansia* spp., (266–268) or BPB species (269,270), is associated with beneficial functions. On the other hand, other bacteria taxa are more frequently identified in pathological conditions, like *Fusobacterium nucleatum*, which appears enriched in colorectal cancer (271) together with other commensal microorganisms with a potential malignancy (pathobionts) (272). Although there is still some controversy over it, the characteristics that set a **eubiotic**

(healthy) microbiota seem to include stability (273), high gene richness or diversity (274) and the dominance of commensal bacteria considered advantageous (275,276).

In view of the importance of a balanced intestinal microbiome and an adequate barrier function in host's homeostasis and fitness, considering information from the host's microbial ecosystem could greatly improve the understanding of processes in health and disease. Hence, researchers must be encouraged to explore the microbiome in future clinical trials (277).

4. Major Modulators of the Gut Microbiota: A Focus on Diet

Generally, the composition of the core GM is pretty stable within adult individuals because of the existence of mechanisms that keep its community steady (198), with some exceptions such as pregnancy (278) or pathological processes (279). The ability to persist and tolerate insults and aggressions is named "*resilience*", concept that was deeply introduced by C.S. Holling in 1973 (280). Briefly, the resilience refers to the capacity of the GM to restore its initial state following a perturbation (281). Given the involvement of the GM in the host's health (described above), this property may be considered when trying to readjust its composition or functioning (282,283).

The host genome can partly determine the composition of the microbiota (194,197), and the ethnic group also has a strong influence that is difficult to dissipate with exogenous factors (230). Nevertheless, many environmental factors can modulate the structure and functions of the GM (254) in a more decisive way than the host's genome (277). These agents include early life events (251), such as the delivery mode (247) or breastfeeding (249), and other general or lifestyle characteristics like age (284), geography (201), adulthood diet (285), physical activity (286,287), psychological stress (238), sleep quality (240) and host's circadian clock (235). Additional elements are the exposure to xenobiotics (288), such as drugs (289) or antibiotics (199,290), or surgical procedures like bypass surgery (291) (**Figure 5, page 44**).

By far, diet and dietary patterns are probably the strongest and more powerful determinants shaping the GM (292–294). It seems obvious, since the nutrients to which our resident microbes have access are those incorporated from the host's diet (227). To date, it has been confirmed the influence of the energy density of the diet (226), calories deprivation (295) or food restriction (296) on the intestinal microbes. Besides, dietary patters such as a Mediterranean-style, vegetarian diet (297) or a diet low in FODMAP components (298) can induce profound changes in specific bacteria taxa of the GM. These changes might be mostly explained by the contribution of readily fermentable compounds (293,299) or macronutrients (300,301).

Certain food groups such as soy and soy-derived products (302) or FFs (34,303), and some dietary small components, such as phytonutrients (i.e., polyphenols, glucosinolates) (304,305), dietary metals, tryptophan (306) and other amino acids (229) and vitamins (304), are also recognized beneficial modifiers of the microbiome. On the other hand, non- caloric sweeteners and other food additives showed to induce dysbiosis (307) and compromise the barrier function, for instance by altering TJs' integrity (308).

Further, a recently published work explored the influence of food cooking methods on intestinal microbes (309). Interestingly, the authors confirmed that cooking techniques can greatly modulate the way food impacts the gut, and such effect was particularly significant in plant-derived foods, whose digestibility was improved compared to its raw version.

Besides shifts in dietary pattern or food preferences, the main approaches for inducing changes in the GM involve the application of probiotics, prebiotics, synbiotics and faecal material transplants (FMT) (191,294,310). I will focus on the most studied ones: probiotics and prebiotics, previously introduced in **Chapter 1**.

Probiotics exert numerous benefits on the consumer, most of them through their action on the resident microbiota (89–91). They can improve the barrier function and adhere to the mucus layer, where they interact with epithelial and immune cells (89), compete with pathogenic bacteria and produce an array of nutritive components and antimicrobial agents (58). The clinical application of probiotics is promising, as many good results have been observed in many pathological processes (90,243,255,311–315). Similarly, prebiotic

components also confer healthy benefits (94). As with probiotics, prebiotic application seems to be useful in the management of many pathological conditions (94,191,263,316,317).

The array of diet-associated factors above mentioned provide us with the opportunity to reseed the gut to combat or prevent diseases. However, owing to the complexity of the human GM, the translation of mechanistic knowledge to clinical practice is probably the most difficult task to carry out and still remains unclear.

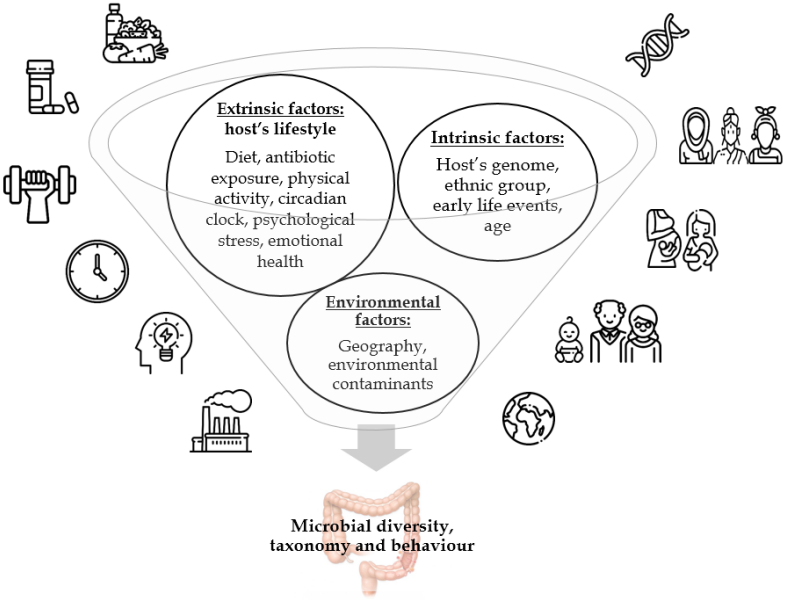


Figure 5. Representation outlining the major contributors involved in the establishment of the gastrointestinal microbiota and its dynamics during life.

Chapter 3

The Role of the Intestinal Microbiota in Diabetes Mellitus

1. Diabetes Mellitus

Diabetes mellitus (DM) is a metabolic disease generally characterized by a chronic presence of elevated glucose levels in the bloodstream (318). Although DM is complex and still not fully understood, the literature has highlighted that there are two main components implicated in the outbreak and progression of the pathology: a compromised insulin sensitivity and/or inefficient β-cell function. The contribution of both insulin resistance (IR) and ineffective activity of pancreatic β-cells in T2D is well documented, however, the interplay between both processes remains partially unknown (319,320). Since insulin is a key hormone for carbohydrate metabolism and energetic regulation, DM involves profound alterations in the metabolism that affect glucose synthesis, transport and oxidation among others (321). Such alterations lead to hyperglycaemia and cause important injuries in many tissues and organs (322). Moreover, when uncontrolled, DM can importantly threaten health causing disability and increasing early mortality (318).

In particular, the pancreatic integrity appears to be an important element to consider. Compelling evidence support the critical role of β-cell functioning in DM since a deficient function of the insulin-producing cells is present in virtually all diabetic individuals at some stage of the disease (322). The occurrence of IR along with such defects in insulin secretion aggravate progressively and develop from certain degree of β-cell dysfunction to a definitive damage. Once reached this point, the insulin secretory islets are unable to keep their activity properly and further complications are likely to arouse (323).

The data on prevalence and incidence of DM is astonishing. In 2018, almost 27 million people were known to present DM in the United States of America, what supposes 8.2% of the population of the country (324). According to the World Health Organization's (WHO) Diabetes Country Profile published in 2016, total prevalence of DM in Spain during that

year was even greater, 9.4% (10.6% and 8.2% in men and women, respectively). Most reports on the topic suggest that the incidence of DM worldwide will increase in the following years, and it is estimated that 642 million adults will be diabetic by 2040 (325).

2. Classification and Epidemiology

Every year the American Diabetes Association (ADA) and its committee of experts provide with updated evidence-based knowledge the classification and diagnosis criteria of DM. In their most recent report published in January 2020 (326), ADA summarized the different diabetes types classified into four main general categories, as presented in **Table 3**.

More than 90% of patients with diabetes present T2D (319,320) and prevalence is greater in male individuals (318). This form of DM has been frequently named "adult- onset diabetes mellitus", however, it is well-known that all forms of DM can happen at all age stages (326). In T2D, the endocrine pancreas cannot properly synthetize insulin to compensate the hyperglycemia due to an inadequate signalling, situation that habitually happens in a context of metabolic and oxidative stress and inflammation (327). In addition, it has been well established the role of overweight in T2D development and progression (321), and it is clear that the presence of high fat depots, especially in the abdominal cavity, exacerbates the predisposition to develop T2D (322,325). Nevertheless, it seems that T2D cannot be attributed solely to an excessive accumulation of body fat, given that not all obese individuals develop T2D, and not all T2D patients present overweight or obesity (321).

Additionally, T1D is generally present in up to 10% of diabetic individuals (318). T1D is an autoimmune disease that affects genetically susceptible subjects and is characterized by the loss of the pancreatic functionality because of the development of autoantibodies against β-cells. Consequently, this situation would eventually cause a residual, if any, insulin secretion (260). T1D affected individuals must use an exogenous source of insulin or alternative treatments to compensate the insufficient production of insulin (328).

Gestational diabetes mellitus (GDM) is the third DM type in prevalence, and occurs in 2-6% of the pregnancies in European countries (329). It is diagnosed during the perinatal period and generally disappears post-partum, however, the risk for suffering T2D in future is increased in women with GDM (325) and, although it remains to be confirmed, it seems that newborns present a greater risk for obesity, T2D and other problems in adulthood (330). Epidemiological studies in a large number of countries have indicated that there is a strong ethnic component in the risk for developing GDM, whose prevalence is greater in Asian and Native-American individuals (331). Further risk factors are family history of GDM or other forms of DM, age and body weight among others (318).

Table 3. Classification of diabetes mellitus according to the American Diabetes Association´s (ADA) latest report (326)**.**

Types	Causes
Type 1 diabetes (insulin- dependent, latent autoimmune diabetes in adults (LADA))	Partial or total deficiency of insulin production originated by an autoimmune destruction of pancreatic β-cells, main responsible for insulin synthesis.
Type 2 diabetes (non- insulin dependent, adults-onset diabetes)	Insulin deficiency that limits insulin's activity. Mostly accompanied by insulin resistance in a context of overweight or obesity.
Gestational diabetes	Diabetes occurring during the second/third trimester of pregnancy, non-diagnosed before gestation. Most cases resolve with delivery.
Other types	Diabetes originated by other causes: monogenic diabetes syndromes, diseases of the exocrine pancreas or diabetes induced by drugs or chemicals among others.

3. Diagnosis

Due to the great differences among the DM types and the importance of timely treatments, an accurate diagnosis is necessary to ensure adequate management of DM. Nevertheless, owing to similitudes among DM types and the nature of the diagnostic tests, in many cases the diagnosis into one class of DM is a difficult task (319). With the objective to guarantee an accurate diagnosis, well-defined and standardized methods have been developed (319). To date, the indicated diagnosis for DM is based on circulating glucose or glycated haemoglobin A1C (HbA1C) (319). The standard criteria includes the analysis of fasting plasma glucose (FPG) levels, that measures circulating glucose concentration in fasting conditions (absence of caloric intake for ≥8 hours), or the oral glucose tolerance tests (OGTT), that refers to glycemia 2 hours after a challenge with an aqueous solution containing the equivalent of 75 g anhydrous glucose. Other criteria relies on the levels of HbA1C (expressed as percentage) (326), that is considered a good alternative diagnostic approach since it reveals the average glycemia levels in the past 2-4 months (332,333).

These same criteria are also helpful for the screening of individuals with an increased risk for developing DM, commonly referred as prediabetic subjects (319). **Prediabetes** itself is not a pathological stage but is a clear marker for the identification of subjects at risk. It has a significant clinical relevance and allows the implementation of strategies to avoid, delay or attenuate the diabetic condition (321,326). Nevertheless, up to 10% of cases will eventually become overtly diabetic (323). The criteria for diagnosis of DM and prediabetes are summarized in **Table 4.**

An early diagnosis is of pivotal importance for the right management of the disease, and it seems to be especially important in prediabetes, given that preventive strategies could dramatically reduce the chances to develop DM (325,328).

Table 4. Criteria for the diagnosis of prediabetes and diabetes based on the American Diabetes Association´s (ADA) latest report (326).

Method	Method Description	Range for Diagnosis of Prediabetes	Range for Diagnosis of Diabetes Mellitus
Fasting plasma glucose (FPG) value	Levels of circulating glucose in fasting conditions (≥8h fasting)	100 mg/dL to 125 mg/dL or 5.6 mmol/L to 6.9 mmol/L	≥126 mg/dl or 7.0 mmol/L
Oral glucose tolerance test (OGTT) value	Levels of circulating glucose 2 h following a challenge with a solution containing 75 g of glucose	140 mg/dL to 199 mg/dL or 7.8 mmol/L to 11.0 mmol/L	≥200 mg/dl or 11.1 mmol/L
Levels of glycated haemoglobin A1C (HbA1C)	Percentage of circulating HbA1C	5.7% to 6.4% or 39 mmol/mol to 47 mmol/mol	≥ 6.5% or 48 mmol/mol
Others	-	-	Random plasma glucose levels over 200 mg/dL or 11.1 mmol/L, accompanied by clinical manifestation of a hyperglycaemic state

4. Symptomatology and Complications

Clinical outcomes in DM are highly heterogeneous. They manifest over time and are hugely connected with the glycemic control (334). In some cases, diabetic individuals do not present evident symptoms of the diabetic disease, for instance in the early stages of T2D due to the progressive development of the pathology (335). This lack of evident symptomatology explains why many people are diagnosed quite later than the onset of the disease (318).

Considering that the presence of peripheral IR importantly reduces insulin action, the whole body is affected, and particularly those glucose-utilizing tissues and organs like skeletal muscle, adipose tissue, liver and heart (318). Among the diabetes-related discomforts we commonly find the characteristic diabetic triad (polyuria, polyphagia and

polydipsia), and loss of weight is quite frequently as well (318). Long-term uncontrolled diabetes causes problems mostly in eyes, kidneys, heart or blood vessels, that led to microvascular and macrovascular complications such as retinopathy, nephropathy and atherosclerotic and cardiovascular problems (319). Notably, DM is considered a great risk factor for coronary heart disease (321) and one of the main causes of renal insufficiency (336).

In subjects presenting T1D, besides the aforementioned complications, hypoglycemic episodes are the main problems (337). Hypoglycemia, that can also occur in T2D (338), is caused by a hyperinsulinemic state, frequently associated to inadequate treatment's prescription or monitoring (337). Some serious health problems associated to this state are stupor and coma, whose complications can cause ketoacidosis, nonketotic hyperosmolar syndrome and even death (318,324,339,340).

5. Experimental Models for Type 1 and Type 2 Diabetes

Because of the high incidence and prevalence of DM, and the great impact the pathology has on patients, strenuous efforts have been made to better understand the disease. For that purpose, numerous preclinical trials on animal models have been performed to shed some light on the pathogenesis of the disease and explore new treatments.

Different animal models can be found for the study of T2D according to the selected method for "developing" the diabetic condition. Some models develop diabetes spontaneously or are genetically manipulated, for instance transgenic mutants or knock-out mice like glucokinase gene locus transgenic mice (341). Other T2D animal models become diabetic following a dietary modulation, exposition to chemicals or a surgery procedure (341). Among T2D models, rodent models with defective leptine or leptine-receptor are the most used. They belong to the group that develop T2D spontaneously, and some examples are ob/ob mice, Zucker fatty rat, Zucker diabetic fatty rat (ZDF) or JCR:LA-cp rat (342). Particularly, the ZDF rat is widely accepted and used for experimental studies on T2D. Besides presenting a defective receptor of the leptin, this model has an intensified

destruction of β-cell islets (343) and deep histological changes in the pancreatic tissue (344). The disease generally shows up within 8-10 weeks in male animals (343), and when T2D is established it causes clinical manifestations like obesity, insulin resistance and dyslipidemia (342). ZDF rats also present other diabetes-related complications like pancreatic and renal failure, retinopathies or neural complications among others (344).

Similarly, for the study of T1D different murine models have been developed. Among them, Non Obese Diabetic mouse (NOD) model and Bio-breeding rat (BB) model are probably the most frequent used models, especially the former, given the great similarities this model shares with human T1D (345,346). Studies performed on NOD mice, that develop diabetes spontaneously, provided with priceless knowledge on the genetic background and ethiology of T1D, as well as on major environmental factors that modulate the development of the disease (347).

6. Risk Factors

A combination of both genetic background and exposure to environmental agents is implicated in the development of DM (318). Some exogenous factors that prone to the prediabetes stage, and hence to DM, include overweight, age and a sedentary lifestyle (323). Importantly, there is a clear role for social and economic development in the risk for DM, since developing countries are specially affected (318).

Concerning T2D, it presents a stronger and more solid genetic background than T1D, however, it has yet to be fully elucidated (319). Up to now, abnormalities in more than 70 loci have been identified by genome wide association studies (GWAS) (318), but only a low number of identified genes can predict the disease (327). Some of these genes are implicated in the function of β-cell population (320,322), inflammation or stress (321). Other factors known to change the risk for T2D are age or ethnic group (326). Nevertheless, it is undeniable that overweight, an unbalanced diet and the lack of physical activity are major causative agents in the diabetic conditions (325). Abdominal fat is an important marker for the risk for dysglycemia (321) and is specially linked to insulin resistance

(327,348). Further, regular dietary habits and energy intake (3,15,349–351), physical activity (352,353), or their combination (354), can have a protective or causative effect on the development of T2D. Additionally, the consumption of certain medications such as glucocorticoids or thiazide diuretics have been associated to an increased risk for developing T2D as well (326). In the last years, an increasing number of scientific publications have called attention to the role of novel risk factors for T2D such as walkability of the residential area (355,356), air pollution (357), endocrine disruptors (358) and intestinal dysbiosis (317). The later one, that has been considered a major epigenetic factor for T2D and other metabolic disorders (359), will be explained in more detail below.

In relation to T1D, up to 40 susceptibility regions of the human genome have been associated with the risk for developing the disease (318). Although the pathology appears in a context of genetic susceptibility, a group of exogenous agents seem to contribute to the transition from susceptibility to autoimmunity against the pancreatic islets and hence development of T1D. Early life events such as rapid growth, obesity during childhood or the breastfeeding duration showed some association with the risk for developing T1D (360). In addition, a proinflammatory status (361), infections of bacterial or viral origin, or disturbances of intestinal IS exacerbated with certain dietary components may be contributing agents (328,362). Intimately related to the later, the role of gut permeability and intestinal microbiota is also well documented in T1D (216).

Some of the aforementioned risk factors are unchangeable, however, others can be easily modified and this opens a window of opportunity to prevent or delay diabetes onset.

7. Rationale for Evaluating the Gut Microbiome in Type 1 and Type 2 Diabetes

As it has been anticipated in the previous paragraphs, recent evidence suggests that there is a role for both gut integrity and gut microbes in the development and progression of T1D and T2D. **Figure 6 (page 54)** offers a graphic explanation of how the gastrointestinal

ecosystem mediates in the progression of both pathologies. In brief, when a dysbiotic state predominates in the gut microbiota, the gut barrier function is compromised because of alterations in the mucus layers and the immune response (363). This situation can lead to an increased gut permeability, altering the transport of molecules and favouring the entrance of particles towards the systemic circulation (254,364). Among these particles, LPS has been extensively studied for its role on glucose homeostasis, as it is intimately associated to the inflammation and altered metabolic state underlying T2D (230,365). When the gut permeability is compromised (194), LPS can reach the systemic circulation, cause metabolic endotoxemia and play a causative role in the pathogenesis of T2D and its related metabolic alterations (225,366). Additionally, most published reviews on the topic agree that functions of the GM associated to energy storage and metabolism, food digestion, regulation of the inflammatory tone and insulin resistance may explain the intimate link between the intestinal microbial ecosystem and T2D (317,367).

Evidence from the existence of a discordant microbiota between diabetic subjects and healthy counterparts supports the putative correlation between dysbiosis and DM. A metagenome-wide association study (MGWAS) on 147 Chinese individuals, 71 of them presenting T2D, revealed differential characteristics of the GM in healthy and diabetic individuals. These include a decrease in species related to the production of butyrate, an enrichment in some mucin-degrading species and pathogenic bacteria, and also enrichment in pathways associated to oxidative stress resistance and sulphate reduction in T2D subjects (368). Another MGWAS carried out in a Chinese population also reported a greater presence of markers of virulence and antibiotic resistance in T2D individuals (369). Interestingly, this study and a previous study carried out in a group of European individuals (370) observed a higher abundance of species of the *Lactobacillus* genus in T2D subjects, and it was hypothesized to be caused by a greater accessibility to nutrients such as glucose. Another features of the GM associated to T2D include a greater abundance of Proteobacteria and certain bacteria taxa, such as *Lactobacillus gasseri* and *Streptococcus mutans*, a higher expression of genes that lead to a proinflammatory status, a lower frequency of genes implicated in the production of some vitamins (366) and also a different profile of antimicrobial peptides and pancreatic enzymes in faecal samples (371).

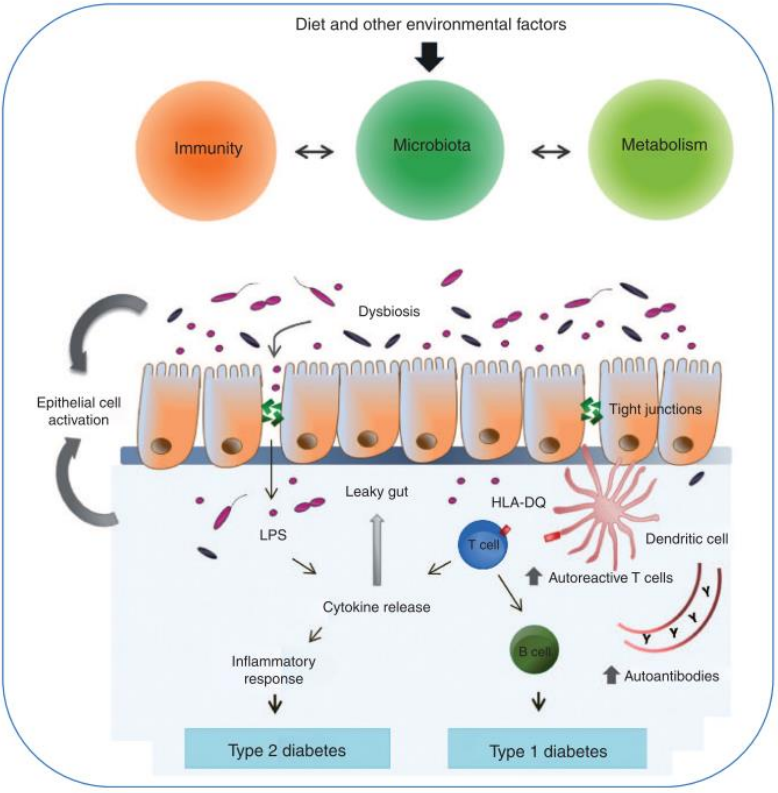


Figure 6. Proposed mechanistic explanation for the connection between the gastrointestinal microbiota, type 1 diabetes and type 2 diabetes (From (363)).

In general, a higher diversity of the gut microbiota is associated with a better insulin sensitivity (372). On the other hand, subjects with IR present greater serum levels of branched-chain amino acids, and it was found that their microbiota presented an enrichment in pathways associated to their synthesis, and conversely a decrease in their transport system (373). Other factors that point towards an intestinal origin for T2D include the fact that exposition to antibiotics may be associated with a greater risk for becoming diabetic (374), and that some pharmacological treatments for T2D cause reduction in circulating LPS (230) and shifts in the abundance of specific bacteria

taxa (192). The later may partially explain the improvement in the disease management following their administration.

Besides T2D, gestational diabetes seems to correlate with certain features in the GM as well. A study comparing 50 women with gestational diabetes and 157 healthy pregnant women found structural differences in the microbial diversity of their GM that involved enrichment and lessening in certain bacteria species. Further, the diabetic microbiota also presented certain similitudes to GM in situations of metabolic alterations, and such changes persisted for some months following delivery (329).

In a like manner, it is increasingly accepted the role of gut microbes in the pathogenesis and progression of T1D (346,359,375). In particular, this may be explained by the implications of the GM for the control of the innate inflammatory response (290,360). When communication between the epithelial cells, the mucosal IS and the GM fails, undesired events occur and increase the risk for sickness (220,254). Studies with individuals with T1D revealed that their GM exhibit characteristic properties, such as a bacterial and fungal dysbiosis and intestinal inflammation (376). What is more, some of the differences between diabetic and healthy subjects were linked to the presence of anti-islet cells autoantibodies (377). Fortunatelly, certain dietary intervention could be helpful to counteract such disturbances. To illustrate, results from preclinical studies suggest that dietary modulations aimed to increase the levels of SCFAs and more specifically acetate and butyrate, may be useful for T1D prevention (359).

8. Therapy

Because of DM is a lifelong burdensome condition entailing clinical manifestations, physical constraints, medical costs, social and psychological issues, it is of great importance the development of efficacious and cost-effective therapies. Treatments should be designed according to patient's characteristics, the stage of the disease, the presence of related complications as well as patient's predisposition (334).

Given that they are the two major components implicated in the progression from normoglycemia to overted diabetes, IR and insulin production (348), most therapeutic approaches for T2D focuses on them (320). Conventional treatments for DM contemplate strategies to reduce body weight or body fat by behavioural changes (319), what will also minimize diabetes-related comorbidities (378). Some therapies focused on lifestyle modification such as improving the quality of the diet or reducing sedentary life seem to be effective in preventing or delaying T2D (339). Even though there is a great deal of controversy about them, some dietary interventions considered for treating DM include caloric restriction, modification of the relative contribution of macronutrients in the diet (379–381), changes in the distribution of the meals with different fasting strategies (382) or the consumption of functional foods (97,109), among others. Similarly, different types of physical activity have shown benefits in diabetic individuals (383).

Being that some of the aforementioned approaches may be difficult to accomplish and/or are not always effective (323), it is also contemplated the use of oral agents like hypoglycemic drugs (338), incretin-based therapies (384) and weight loss medication (378). Other strategies include surgeries, such as bariatric surgery or Roux-en-Y gastric bypass (320,331), that have proven to induce significant changes in the GM composition (385,386), or gene therapy, that seems to offer some advantages over other approaches (360). In some cases, it is also necessary to treat diabetes comorbidities like dyslipemia with statins or other lipid-lowering agents (387,388).

Regarding T1D, treatments must contemplate the restoration of the immune tolerance and preservation of the residual activity of β-cell islets as main objectives (328). Given the massive loss of pancreatic function in T1D, most patients need the exogenous administration of insulin or insulin analogues. The administration mode varies from one single dose or injection to continuous infusion using insulin pumps (389). The latter proved to be useful in controlling the individual glycemic status (390) and seems to reduce complications more than the conventional treatment. For that reason, it may present an alternative therapeutic strategy to standard treatments (389). Similarly to T2D, gene therapy has also been contemplated for T1D management, and some studies have

suggested its potential therapeutic use in future (360). Lastly, some authors suggest that the combination of more than one treatment would increase the chances to obtain significant improvements in the management of the disease (328).

Given the growing body of evidence on the role of both gut integrity and intestinal microorganisms in the development and progression of both T1D and T2D (previously described in this chapter), novel microbiota-driven therapeutic approaches have been explored in the last decades.

Regarding T2D, probiotic (391–393), postbiotics (55,394), prebiotics (395), and symbiotic (396,397) therapies, in addition to FMTs (372) or antibiotics (230), have shown to be useful in improving the glycemic control and/or attenuating comorbidities and complications derived from the pathology in many cases. In parallel, research focused on T1D has also shown a potential therapeutic role for probiotics (268,398) and prebiotics (399,400), and a future application of FMTs (400) and postbiotics (401) has been considered as well.

Chapter 4

Probisan®

1. Probisan®

The present doctoral thesis has relied on the study of Probisan®, a plant-based fermented product elaborated with a combination of LAB and yeasts. The product is produced and distributed by the company PentaBiol S.L., located in Esquiroz, Navarra, Spain. According to the International Scientific Association for Probiotics and Prebiotics' (ISAPP) latest expert consensus report on probiotics (88), the product would meet the criteria for "*fermented food with undefined microbial content*". Moreover, due to its formulation, specially the microorganisms intentionally added and the compounds generated during its manufacturing, Probisan® is considered a functional food with postbiotic effects.

2. Uses and Properties

The commercialization of Probisan® is intended for animal feeding and the product is used for the supplementation of fodder in animal farms. The company offers different Probisan® formulations for the production of ruminant, swine, poultry and rabbits, and the recommended dose varies greatly among each animal species, age and weight. To illustrate, focusing on Probisan® formulated for ruminants (used in *Study 1* and *Study 3*), the suggested dosage varies from 3-10 g/day in breastfed animals (lambs-young cattle), 1- 3 kg/tonne of feed in growth or fattening animals, and 1.5-15 g/day for animals during milk production (sheep-dairy cattle).

Many favourable outcomes have been observed with the administration of Probisan® to animals. To start, studies in animals have revealed that Probisan® is effective in improving the digestive function. This has become evident after improvements in the anatomy of the gastrointestinal track with an enhanced intestinal barrier function and an increase in the absorptive surface (swine), and a better ruminal function and fiber digestibility

(ruminants) have been confirmed. In the same line, the use of Probisan® also leds to changes in the GM. It was evidenced by changes in the metabolic activity of the GM, such as a greater production of SCFAs, as well as changes in its composition, evidenced with a greater microbial diversity, enrichments in healthy bacteria taxa, like LAB, and depletion of potential pathogens microorganisms, like *Clostridium* spp. and *E. coli* (ruminants and swine).

The supplementation with Probisan® also caused changes in productivity, for instance showing faster growth and fattening and lower conversion rates, improving eggs and milk quality (i.e., with greater abundance of LAB in milk), allowing a better postpartum recovery and also a decline in the use of medication (ruminants, swine and poultry).

Additionally, the product was able to boost the systemic immune response, with a greater stimulation of the innate IS (swine) and improvements in the host defence against pathogens, illustrated by observed greater levels of immunoglobulin A or the attenuation of the inflammatory response in *ex vivo* infectious challenges with *E. coli* and *Salmonella* LPS (ruminants). Similarly, the supplementation with Probisan® resulted in a reduced incidence of frequent complications and helped with a better recovery (ruminants). (Information provided by Pentabiol S.L., and obtained from different research projects within the framework of the project HEALTHSTOCK, funded by the European Union Research and Innovation Programme Horizon 2020).

Overall, all those benefits mentioned above contribute to an improved health status, and allow for the reduction or elimination of the use of antibiotics, with profound implications for the entire food chain (93). Furthermore, given that antibiotic resistance has become a huge global health burden (402,403), every strategy designed to reduce antibiotic stewardship would imply important savings.

3. Ingredients and Nutritional Profile

The batch of Probisan® utilized in the present doctoral thesis was primarily composed by soya flour, alfalfa meal and malt sprouts along with other minor components like skimmed milk powder. Importantly, a group of selected bacteria and yeast strains (composition protected by trade secret) is integrated with the aforementioned raw materials, and decisively contribute to the nutritional and functional properties of Probisan®.

The caloric density of Probisan® is 467.6 kcal/100 g of product. Carbohydrates account for the 53.1% of the energy, while proteins and fats represent 44.5% and 2.4% of the total energy, respectively. According to producers, the array of bioactive compounds in Probisan® include enzymes (amylases, proteases, lipases and cellulases), volatile organic acids and B-complex vitamins among other molecules.

A culture-based analysis (plate count) performed in our laboratory revealed the quantity of alive microorganisms present in Probisan® (**Table 5**). Additionally, the metagenomic analysis showed that Firmicutes and Proteobacteria are the most predominant phylum (38.7% and 26.7%, respectively), followed by Bacteroidetes (18.3%), Actinobacteria (14.5%) and TM7 (1.8%). At genus level, *Lactobacillus* is the most predominant genera accounting for more than 6% of identified species.

Table 5. Culturable and viable counts in Probisan®.

Target Microorganisms	Media and Conditions	Viable Counts (CFU/g)
Total Bacteria	Tryptic Soy Agar, 37 ºC for 24-48h, aerobiosis	2.0×10^5
Lactobacilli	Man, Rogosa and Sharpe Agar, 37 ºC for 24-48h, anaerobiosis	4.6×10^7
Yeast and Fungi	Potato Dextrose Agar, 25 ºC for 2-4 days, aerobiosis	1.0×10^5

The current formulation of the product Probisan® is slightly different and presents a different cereal substrate.

4. Production and Manufacturing

The complete composition of LAB and yeast species and the production process of Probisan® are protected under industrial property rights. Briefly, the first stage of the production of Probisan® covers the fermentation of a mixture of precultured starter microorganisms in Tryptic Soy Agar (TSA) at 37 ºC. This incubation is maintained until the culture reaches a load of 10^9 microorganisms/mL of media, in an exponential growth phase. Subsequently, this mixture is incorporated to the solid ingredients for a second fermentation process. Lastly, convective air drying is used to reduce moisture content in the final product (**Figure 7**).

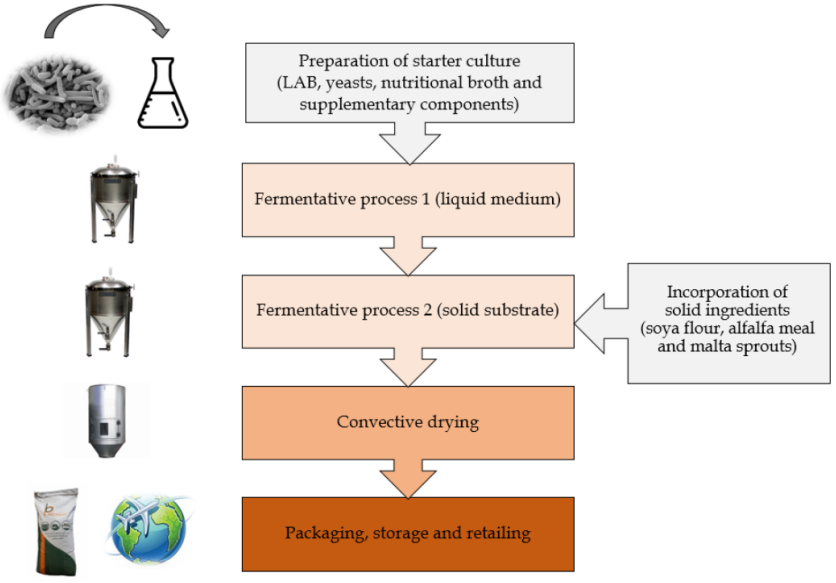


Figure 7. Industrial production of Probisan®.

5. Product Presentation

Probisan® is routinely presented micronized, as a dry granulated product with an appearance similar to fine sawdust, and an average particle size of 0.1 mm (**Figure 8A**). Moisture content is approximately 12.8% and pH is close to 4.4. For its administration to the experimental animals in the *Study 1*, the product required a greater particle size to facilitate food intake by the ZDF rats. For that purpose, minor modifications were performed on the production process, particularly in the drying process. As a result, the product presented a rough aspect and a particle size ranging from 4 to 12 mm (**Figure 8B**).

Figure 8. (A) Illustration of the Probisan® used in the *Study 3* (regular format). (B) Illustration of the Probisan® tested in the ZDF rats model, *Study 1* (granular format).

Further information on the product characteristics and its applications is available on the company's website https://www.pentabiol.es/.

Hypothesis

To date, experimental and clinical evidence support the beneficial effect of fermented food products in human and animal health, contributing with both health promotion and disease protection. The attributes of functional foods depend on their intrinsic characteristics, and in order to extend their beneficial properties during shelf life, it is of great relevance to identify the best storage condition for a given product. In view of this, and considering that fermented foods belong to a very heterogeneous group, personalized research projects are needed to test each product individually.

The hypothesis of the present thesis is twofold,

First, we consider that the administration of a fermented product that has previously offered many health benefits in livestock production (Probisan®) would have some beneficial effects in a model of human disease, specifically the Zucker Diabetic Fatty rat, widely used as animal model of type 2 diabetes. We consider that the supplementation with Probisan® would reduce the incidence and/or side effects of the diabetic disease in the animals. Particularly, we believe that the product would induce such results through the modulation of the gastrointestinal microbiota. If effective, this type of intervention would be a possible therapeutic intervention in clinical practice.

Secondly, we also hypothesize that the storage of Probisan® under different temperature and packaging conditions would induce significant changes in its intrinsic characteristics, and most notably in the load of viable microorganisms. We estimate that low storage temperature and vacuum packaging would greatly protect the microbial community present in Probisan®, what could, to some extent, enlarge its functional properties.

Research Objectives

The **general objective** of the present thesis was to investigate whether the supplementation of the diet with Probisan® could be helpful for the management of type 2 diabetes by protecting against the development of the pathology, improving glucose homeostasis and/or attenuating other manifestations typically found in the context of the disease. In line with this, we also aimed to gain a deeper understanding of the role of the gastrointestinal microbiota in glucose homeostasis, its repercussion in the pathophysiology and microbiota-driven therapies. The third purpose of the thesis was to determine whether there are changes in the fermented product Probisan® throughout its storage and define the most suitable storage conditions for the preservation of its microbial fraction and hence, its functional properties.

In order to achieve the general objectives above mentioned, we established the following **specific objectives:**

1. To study if the administration of Probisan® in an animal model of human type 2 diabetes (ZDF rats) can induce a normoglucemic effect on the animals, and/or attenuate diabetes-related discomforts (*Study 1*).
2. To determine if the early supplementation with Probisan® avoids the onset of the disease (*Study 1*).
3. To review and summarize the start-of-the-art in the management of type 1 diabetes targeted immunotherapy strategies (*Study 2)*.
4. To provide with an updated compendium on the novel treatments that could be considered for treating the autoimmune type 1 diabetes, with a special focus on the resident microbiota (*Study 2*).
5. To investigate whether there are changes in Probisan®'s physicochemical and microbiological profiles throughout its storage, and determine the influence of different storage temperatures and packaging modes on Probisan®'s characteristics (*Study 3*).
6. To identify the best storage condition for maintaining the functional characteristics of Probisan® over time (*Study 3*).

Experimental Design

The present research work was developed into three projects: two experimental studies and one literature review.

The first study (*Study* 1) aimed to explore the effect of Probisan® in the type 2 diabetic phenotype that develops the murine model ZDF. For that purpose, we conducted a pre-clinical experimental study with two groups of ZDF rats, one control group and one treated group, and the latter group received Probisan® for a long period of time (31 weeks). The animal protocol was reviewed and approved by the Institutional Committee on Care and Use of Laboratory Animals (CEEA, University of Navarra; Protocol number CEEA/117-15). During and after the intervention we measured a number of parameters for the evaluation of glucose metabolism and homeostasis, as well as other biochemical and histological variables we considered useful according to the objectives of our studies (fasting blood glucose, glucose tolerance, body weight, body gain, body composition, adipose tissues, serum C-peptide, lipid profile, transaminases, intestinal glucose uptake and faecal microbiota composition). In this study, I was under the animal-trained period and I assisted with the care and maintenance of the animals, with the data collection and with the sacrifice of the animals, I assembled and analysed all the data, and drafted the first version of the manuscript.

The second study (*Study 2*) was performed in collaboration with a group of American researchers with considerable expertise in the field of immunology and immunotherapies, and previous experience with diabetes mellitus, and specifically type 1 diabetes, and the GM. This work revolved around the currently available immunotherapy strategies for treating T1D. The main purpose was to examine immunotherapies for T1D through a narrative review. With that goal in mind, the main scientific databases were searched up and identified records were screened for inclusion. Those selected articles were further examined, and relevant data were extracted. I specially contributed with the section titled "Microbiota Modulation".

In the last study (*Study 3*) we aimed to evaluate the effect of storage conditions on Probisan®'s microbiological and physicochemical characteristics. For that reason, the product was exposed to its regular storage temperature and packaging (room temperature, 22 ºC; and standard packaging, respectively) and also to other conditions that could be achieved during its shipping or storage. The latter include three alternative storage temperature (freezing, −20 ºC; cooling, 4 ºC and high temperature, 37 ºC) and one alternative packaging mode (vacuum packaging). The product was sampled in small packages and was stored under the aforementioned conditions in our laboratory. During the product shelf life (one year), we monitored microbiological and physicochemical attributes of Probisan® stored in different temperature and packaging conditions. We determined counts of total bacteria, LAB and yeasts, moisture content and pH values. I designed the experimental protocols, prepared materials and equipment, and adapted the installations of the laboratory in order to execute the experiments. I also collected and analysed data, and drafted the first version of the manuscript.

Results & Discussion

~ Study 1 ~

A Fermented Food Product Containing Lactic Acid Bacteria Protects ZDF Rats from the Development of Type 2 Diabetes.

~ Study 2 ~

New Insights into Immunotherapy Strategies for Treating Autoimmune Diabetes.

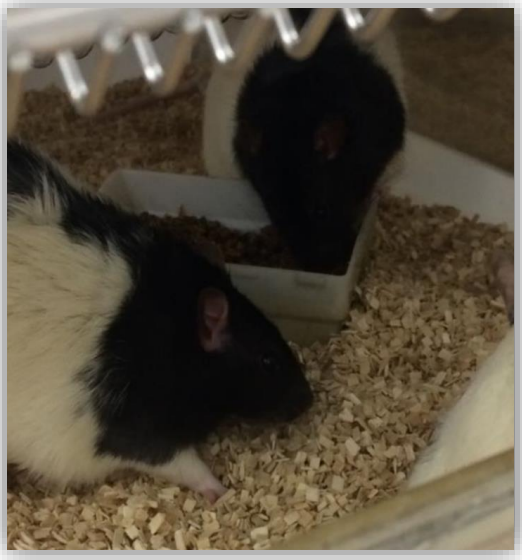
~ Study 3 ~

Influence of Storage Temperature and Packaging on Bacteria and Yeast Viability in a Plant-Based Fermented Food.

~ General Discussion ~

~ Study 1 ~

A Fermented Food Product Containing Lactic Acid Bacteria Protects ZDF Rats from the Development of Type 2 Diabetes.



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Abstract: Type 2 diabetes (T2D) is a complex metabolic disease, which involves a maintained hyperglycemia due to the development of an insulin resistance process. Among multiple risk factors, host intestinal microbiota has received increasing attention in T2D etiology and progression. In the present study, we have explored the effect of long-term supplementation with a non-dairy fermented food product (FFP) in Zucker Diabetic and Fatty (ZDF) rats T2D model. The supplementation with FFP induced an improvement in glucose homeostasis according to the results obtained from fasting blood glucose levels, glucose tolerance test, and pancreatic function. Importantly, a significantly reduced intestinal glucose absorption was found in the FFP-treated rats. Supplemented animals also showed a greater survival suggesting a better health status as a result of the FFP intake. Some dissimilarities have been observed in the gut microbiota population between control and FFP-treated rats, and interestingly a tendency for better cardiometabolic markers values was appreciated in this group. However, no significant differences were observed in body weight, body composition, or food intake between groups. These findings suggest that FFP induced gut microbiota modifications in ZDF rats that improved glucose metabolism and protected from T2D development.

Keywords: diabetes; fermented food; gut microbiota; lactic acid bacteria; postbiotic; probiotic

1. Introduction

Type 2 diabetes (T2D) is characterized by a chronic hyperglycemia preceded by a deranged insulin sensitivity and/or synthesis. This chronic non-communicable disease accounts for the vast majority of cases of diabetes, more than 90% [1], being one of the most prevalent illnesses. Importantly, T2D is associated with over 70% of global deaths [2]. Genetic factors contribution is unlikely to be responsible for the increasing T2D incidence because of the genome stability, although physicians and the scientific community have also focused on environmental factors [3,4]. One possible explanation hypothesizes the role of microorganisms that coexist with us [5]. The gut microbiota (GM) is the bigger reservoir (10^{14} microbes) [5] and is considered an organ because of its crucial metabolic and defense competences [6]. Its anomalous distribution or activity, named dysbiosis [7], has been related to a wide group of illnesses and physiopathological conditions [8]. Specifically, there is evidence that diabetes occurs in association with a compromised gut environment and a large body of research highlights a plausible connection between T2D and GM [9 - 11]. For instance, a compromised abundance of beneficial bacteria [12] or increase of infrequent species [13] have been reported in T2D and obese individuals.

The GM displays some plasticity and different strategies resulted in marked changes. Among the studied approaches, dietary modulation was successful in causing significant changes in the GM [13,14]. Probiotics, prebiotics, symbiotics, and fermented foods are known to confer health benefits on the host by improving GM performance and have been proposed as novel clinical strategies for T2D [6,15,16]. For instance, probiotic supplementation with *Lactobacillus* G15 and Q14 showed improved glucose tolerance in streptozotocin (STZ)-induced type 2 diabetic rats [17]. Human intervention studies have also shown good results. A crossover trial with prediabetic individuals reported a reduced insulin resistance after kimchi consumption [18], and a randomized controlled trial in T2D subjects supplemented with a fermented milk with the probiotic Lactobacillus casei strain Shirota showed a healthier gut ecosystem with strengthened gut barrier function along with modulation of microbial communities [19].

The most widespread fermented products are dairy products like yogurt, kefir, or dahi.

Nonetheless, other food matrixes like fruits, vegetables, or cereals have also been studied and showed many beneficial effects on health [20]. Indeed, some attractive advantages over dairy products have been described for them [21]. In the present study we have tested the effectiveness of a non-dairy fermented food product (FFP) in preventing the T2D and obese phenotype developed by the Zucker diabetic fatty (ZDF) rat model. This murine model presents a mutation in the leptin receptor accompanied with an enhanced β-cell destruction and impaired glucose homeostasis, and is a widely used model for T2D studies [22,23]. Previous research reported the effectiveness of probiotics in the attenuation of the diabetic and obese phenotype in ZDF rat model [24] and other rodent models [25,26]. However, few researchers have tested the efficiency of fermented food on the diabetic phenotype and previous works have only focused on dairy products [27]. On top of this, the literature on probiotic microorganisms indicates that the functional attributes of the cells are to a large degree dependent on the strain [28,29]. Therefore, an individual characterization should be performed for each study product. Our fermented food product derives from a fermented feed, which has previously demonstrated the ability to improve health and wellness in farm animals (HEALTHSTOCK, Ref.733627; more information available in https://cordis.europa.eu/project/rcn/206082/factsheet/es). Consequently, FFP has been used in a controlled in vivo study (ZDF rat model) in order to demonstrate its benefit in glycemic control and in comorbidities derived from hyperglycemia.

2. Materials and Methods

2.1. Product Description

The fermented food product tested is a plant-based food product primarily composed by soya flour, alfalfa meal, and barley sprouts along with other minor components (including skimmed milk powder). The FFP is defined as a non-probiotic product classified as fermented food [29]. During the production process a combination of specific LABs and non-bitter beer yeast is incorporated to the raw materials and a classical fermentation process is performed. The FFP has been produced using standard culture medium Tryptic Soya Agar (TSA) in microaerophilic conditions at 37 ºC until microorganisms's

concentration achieve at least 10^9 microorganisms/mL in an exponential growth phase or close to the stationary phase. The manufacturing as well as the pool of microorganisms intentionally added are responsible for the viable microorganisms and composition of the final product (**Tables S1** and **S2**). The FFP is presented as a dry granulated product, with an average particle size ranging from 4 to 12 mm (**Figure S1**) with a moisture content of 12.8% and a pH of 4.4. The metagenomics analysis revealed that Firmicutes is the most predominant phylum (38.7%), followed by Proteobacteria (26.7%), Bacteroidetes (18.3%), Actinobacteria (14.5%), and lately TM7 (1.8%). At genus level, *Lactobacillus* are the most predominant accounting for more than 6% of identified species.

2.2. Animals and Experimental Design

Eleven weeks-old male ZDF rats (n = 16) (Charles River Laboratories) were acclimated for five weeks and housed in a controlled environment (a room with constant temperature and humidity under a 12:12 h light-dark cycle) with ad libitum access to food (standard rodent chow) and water. Animals were randomly divided and allocated into two groups: A control group (C group) (n = 8) and a group supplemented with FFP (T group) (n = 8). ZDF rats were housed at four animals per cage. After the acclimatization period, all animals were given hypercaloric diet (HD) (TD.06416; Envigo) until the end of the study, which lasted 31 weeks (see composition of HD in **Table S2**). The T group was additionally fed with FFP (200 g per cage and week). See the experimental design scheme (**Figure S2**). The glucose uptake assay, the insulin positive cell quantification such as the analysis of the microbiota were analyzed by blinded investigators.

Animal procedures were performed in accordance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources, Commission on Life Science, National Research Council, and published by the National Academy Press, revised 1996. All animal procedures were approved by the Institutional Committee on Care and Use of Laboratory Animals (CEEA, University

of Navarra) (Protocol number: CEEA/117-15).

2.3. Fasting Blood Glucose and Intraperitoneal Glucose Tolerance Test

Animals were fasted 24 h and blood samples were collected from the tip of the tail vein in order to determine blood glucose levels by using a glucometer (Accu-chek Aviva, Roche, Basel, Switzerland). Fasting blood glucose (FBG) was recorded once a week.

For glucose tolerance test (GTT) determination, 24 h fasted animals received glucose (Baxter, Valencia) intraperitoneally (1.5 g/kg of body weight) and glycemia was determined as described for FBG at different time points (baseline, 20, 40, 60, 90, 120, and 150 min) after glucose injection. GTT was conducted at one and two months after the start of the study. The area under the curve (AUC) of glucose values was assessed for each group from 0 to 150 min post glucose injection.

2.4. Body Weight, Food Intake and Body Composition

Body weight (BW) was measured once a week with an electronic balance. HD food intake was monitored weekly for 12 weeks. The weekly HD intake mean was estimated for the two experimental groups as grams per week and animal. Also, body composition was determined for each group (n = 4 in C group; n = 5 in T group) using nuclear magnetic resonance (EchoMRI, EchoMedical Systems, Houston, TX, USA) at the end of the study.

2.5. Analysis of Functional Properties: C-peptide Synthesis and Intestinal Glucose Uptake Assays

Blood C-peptide concentration (ng mL^{-1}) was quantified with a commercial C- Peptide ELISA kit (Crystal Chem Europe) at time points four and seven months from the beginning of the study. HOMA-IR and HOMA-β were determined at the end of the study. HOMA-IR was calculated by the formula: HOMA-IR = serum C-peptide (ng mL^{-1})*blood glucose

(mmol L^{-1})/22.5; and HOMA-β was calculated by the formula: HOMA-β = 20*serum C-peptide (ng mL^{-1})/(blood glucose (mmol L^{-1}) − 0.35).

The effects of in vivo FFP supplementation on the uptake of α-Methyl-D-glucoside (α-MG), a SGLT-1 specific substrate, were determined on everted jejunal rings obtained from the animals as previously described [30]. Briefly, at the end of the study, animals were sacrificed, the jejunum obtained, and groups of six rings were incubated at 37 ºC for 15 min under continuous shaking in Krebs-Ringer-Tris (KRT) solution gassed with O_2. The solution contained 1 mM α-MG and 0.0025 µCi mL^{-1} of [14C] α-MG (Ge Healthcare, Little Chalfont, UK). At the end of the incubation period, rings were removed from the medium, weighed, and the accumulated substrate was extracted from the rings for 15 h in 0.1 M HNO_3 at 4 ºC. Finally, duplicate aliquot samples were taken for liquid scintillation counting. α-MG uptake was estimated from the relationship between the counts per minute recorded for the incubation medium and the counts per minute obtained for the HNO_3 aliquots and expressed as micromoles of α-MG per gram of wet weight (w.w.) per 15 min.

2.6. Lipid Profile and Hepatotoxicity Markers

In order to determine the lipid profile, fasting blood samples were extracted from the dorsal pedal vein under anesthesia (5% isoflurane in oxygen) at baseline, two, four, and seven months of the study. Samples were centrifuged 15 min at 13,000 rpm and stored at −80 ºC for biochemical analysis. Also, serum total cholesterol (TC), high-density lipoprotein (HDL- C), low-density lipoprotein (LDL-C), triglycerides (TG) and aspartate and alanine amino-transferases (AST and ALT, respectively) were analyzed (Cobas c311 analyzer, Roche, Basel, Switzerland). The atherogenic index (AI) was estimated using the formula log(TG/HDL-C) as previously described [31].

2.7. Tissue Collection and Histological Analysis

After 31 weeks of study, o/n fasting animals were sacrificed by decapitation. The pancreas and fat tissues (retroperitoneal, epididymal, mesenteric, subcutaneous, and brown fat)

were immediately removed, weighted, and fixed in 10% buffered formalin.

Fixed pancreas samples were embedded in paraffin blocks, cut at a thickness of 3 µm and analyzed by immunohistochemistry (n = 4 for each group). Immunolabelling was performed with an antibody against insulin (dilution 1:8000, A0564 Dako) and a secondary antibody labeled with HRP (dilution 1:100, P0141 Dako). All sections were observed under an optical microscope using the 10× objective lens (Olympus CH, Shinjuku, Japan) and insulin positive cells were counted. Nine serial sections were analyzed for each pancreas.

The total area (mm^2) of the analyzed sections was calculated. For this purpose, slides containing the stained histological samples were digitized (APERIO CS2, Leica Biosystems, San Diego, CA, USA) and images were analyzed using the ImageJ 1.52 software. The results of the quantification are shown as insulin-positive cells per pancreas area (insulin positive cells/mm^2) at the end of the study.

2.8. Faecal Microbiota Analysis

Rat feces were collected at six months of study and immediately frozen at −80 ºC for the purpose of metagenomic analysis. 16S rRNA sequences obtained were filtered following quality criteria of the OTU processing pipeline LotuS (release 1.58) [32]. This pipeline includes UPARSE de novo sequence clustering [33], removal of chimeric sequences and phix contaminants for the identification of operational taxonomic units (OTUs), and OTU abundance matrix generation. Finally, taxonomy was assigned using BLAST [34] and HITdb [35] achieving up to species-level sensitivity. The abundance matrices were first filtered and then normalized in R/Bioconductor [36] at each classification level: OTU, species, genus, family, order, class, and phylum. Briefly, taxa were discarded for future analysis when less than four reads were obtained in more than 50% of the samples of both experimental conditions, and a global normalization was performed using the library size as a correcting factor and log2 data transformation. Linear models for microarray data (LIMMA) [37] was used to identify taxa with significant differential abundance between experimental conditions. The selection criteria was based on an FDR cut-off (FDR < 0.05).

Further clustering analyses and graphical representations were performed using R/Bioconductor [36].

2.9. Statistical Analysis

Statistical analysis was performed with the SPSS 22.0 for windows software package. Normality and variances homogeneity were tested with the Shapiro–Wilk and Levene tests, respectively. For values showing normal distribution, comparisons were carried out using unpaired and paired Student's T-test and in case of non-normal distribution, U-Mann-Whitney test. Results are expressed as mean ± standard deviation (SD). Statistical significance was set at $p < 0.05$ and $p < 0.01$ was considered as highly significant.

3. Results

3.1. FFP Supplementation Leads to Lower Fasting Blood Glucose Levels and Improves Glucose Tolerance

In order to determine the effectiveness of FFP supplementation to control blood glucose levels in ZDF rats supplemented with HD we have performed a FBG determination on ZDF rats fasted during 24 h. No significant differences between groups were observed in FBG values basally (**Figure 1A**). However, after four weeks of supplementation, animals in the T group exhibited lower FBG values than animals in the C group (8.0 ± 1.8 versus 12.0 ± 2.7 mmol L^{-1}, respectively; $p = 0.004$). Statistical significant differences between groups were also found in week six ($p = 0.038$), week 14 ($p = 0.013$), week 16 ($p = 0.013$), week 21 ($p = 0.005$), week 22 ($p = 0.026$), week 24 ($p = 0.001$), week 27 ($p = 0.03$), week 28 ($p = 0.001$), and week 31 ($p = 0.016$) as represented in **Figure 1A**. Initial and final FBG values were compared in the C group showing no statistical significant differences, despite the observed differences were very high ($p = 0.125$); on the other hand, initial and final FBG values in the T group are comparable and no statistically significant differences were found ($p = 0.625$) (**Figure 1B**). Although, one-month period was not enough to display significant

differences in GTT between groups (data not shown), after two months of supplementation, the T group showed lower glycemic values versus the C group although not all the time points showed significance (**Figure 1C**). Statistically significant differences were found at GTT time point 20 min (p = 0.034), 40 min (p = 0.007) and 120 min (p = 0.038) time points of the GTT. Glucose AUC showed higher values in the C group with a p value in the limit of significance (p = 0.05)(**Figure 1D**). These findings suggest that rats supplemented with the FFP exhibited a better glucose metabolism control than those fed exclusively with HD (C group).

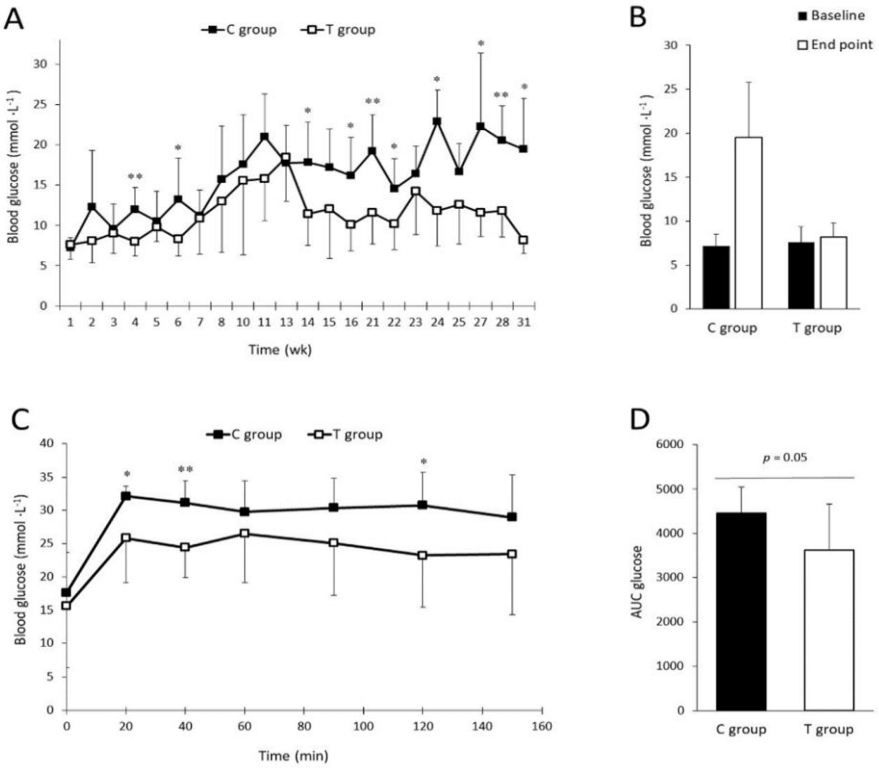

Figure 1. Effect of the FFP on Glucose Metabolism. (A) Weekly FBG progression in ZDF. Animals in T group showed lower FBG mean values than animals in C group during the study. Statistically significant differences were found at 4, 6, 14, 16, 21, 22, 24, 27, 28 and 31 weeks ($p = 0.004$, $p = 0.038$, $p = 0.013$, $p = 0.013$, $p = 0.005$, $p = 0.026$, $p = 0.001$, $p = 0.030$, $p = 0.001$ and $p = 0.016$, respectively). (B) Bar plots represent basal and end point FBG values in the groups. No statistical significant differences were found between basal and end point FBG values in any groups (7.2 ± 1.3 versus 19.5 ± 6.3 mmol L-1 at 0 and 31 weeks respectively in C group; 7.6 ± 1.8 versus 8.2 ± 1.6 mmol L^{-1} at 0 and 31 weeks respectively in T group). (C) Two-months GTT curve. The GTT curve, after 2 months of FFP supplementation, showed lower blood glucose levels and statistical significant differences between groups were found at 20, 40 and 120 minutes ($p = 0.034$, $p = 0.007$ and $p = 0.038$, respectively). (D) AUC plot. The AUC value was lower in T group (3622.5 ± 1040.4) compared to C group (4454.0 ± 590.9) although the p value obtained was in the limit of significance ($p = 0.05$). Values are expressed as mean ± SD. * p <0.05, ** p <0.01, *** p <0.001.

3.2. Body Weight, Body Composition, and Food Intake after FFP Consumption

Initial body weight values were similar between groups (p = 0.610) and BW increase was steady and similar within the groups during the first eight weeks of study, time at which different trends were observed between groups. From week eight onward, the T group continued gaining weight whereas the C group did not increase BW and even a weight loss was observed at the end of the study (**Figure 2A**).

Regarding the BW gain (difference between end point and basal BW values), statistically significant differences were observed in the T group (p = 0.031), while the C group did not experiment statistical significant changes over time (p = 0.625) (**Figure 2B**).

No statistically significant differences were found in HD food consumption between both groups (p = 0.413) (**Figure 2C**). Interestingly, data on food intake differs from the outcomes found in BW, which indicates that animals supplemented with the FFP showed a greater BW gain.

Body composition was evaluated before sacrifice and despite the asymmetry of BW found at the end of the study, no statistically significant differences within groups were assessed in fat mass percentage (p = 0.630). Both groups also exhibited similar mean values of lean mass (p = 0.641) and other tissues relative percentage (p = 0.947) (**Figure S3**). These results suggest that the supplementation with the FFP did not alter body composition in ZDF rats fed with HD. Supporting the previous presumption, the weights of retroperitoneal, epididymal, mesenteric, subcutaneous, and brown fat mass collected at sacrifice did not statistically differ between experimental groups (**Table S3**).

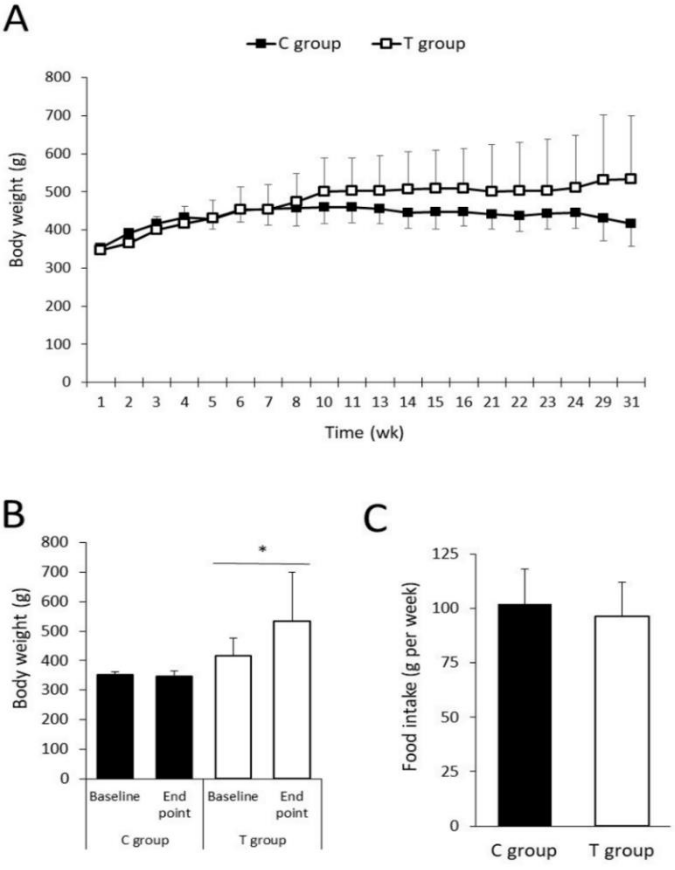

Figure 2. Body Weight Progression, BW Gain and Average Food Intake During the Intervention. No statistical significant differences were observed in BW (A) between the experimental groups in spite of their divergent growing tendencies. BW gain (B) was statistically significant in T group (346.0 ± 18.4 versus 533.5 ± 165.8 g at baseline and at the end point respectively; p = 0.031) but no statistical significant differences were found in C group (352.3 ± 9.9 versus 416.8 ± 60.3 g at baseline and at the end point respectively; p = 0.625). The FFP administration did not alter food intake (C), which was comparable in both groups (101.9 ± 16.2 versus 96.5 ± 15.7 g in C and T groups respectively; p = 0.413). Values are expressed as mean ± SD. *p < 0.05.

3.3. FFP Preserves Normal Metabolic and Biochemical Parameters

Comparisons of lipid profile and hepatotoxicity markers between and within groups are available in **Table S4**. No statistically significant differences were found at baseline in any of the explored parameters between both experimental groups, except for LDL-C (p = 0.021) and TG mean values (p = 0.025). During the study both groups experimented an increase in LDL-C values and the magnitude was greater in the C group than in the T group (p = 0.001 and p = 0.023, respectively). Although baseline TG levels were significantly lower in the C group (p = 0.025), a statistically significant increase was observed for the same group after seven months of study (p = 0.043) while T group remained unchanged. Indeed, significant differences were appreciated between groups at the end of the trial (p = 0.01). Somehow the FFP could restore and normalize TG values. Regarding TC, both C and T groups showed the same tendency of increase at the end of the study (p = 0.00 and p = 0.011, respectively) and the same response was found in LDL- C (p = 0.01 and p = 0.023 in the C group and T group, respectively). Taken together, pairwise comparisons between all of the follow-up time points revealed that at the end of the study both groups showed significantly greater serum levels of TC, LDL-C, and HDL.

With respect to the liver function and the stress induced by the HD, serum AST and ALT levels were determined along the intervention as well. For both parameters, statistically significant differences were found between T and C groups at two months of study, when a peak on serum AST and ALT was observed: The T group showed significant lower values of both AST and ALT (p = 0.001 and p = 0.019, respectively). After the aforementioned peak, transaminase levels were normalized, and such reduction was more pronounced in the group supplemented with FFP (**Figure 3A**). The AI showed statistically significant differences between groups at baseline (p = 0.023) and at the end of the study (p = 0.020) (**Figure 3B**). The T group presented a tendency with a better health status in the T group and when pairwise comparison was made, the treated group exhibited an improved and reduced AI at the end of the study in contrast with its baseline value (p = 0.027). Regardless the tendency observed in **Figure 3B** the C group did not present marked differences on AI over time.

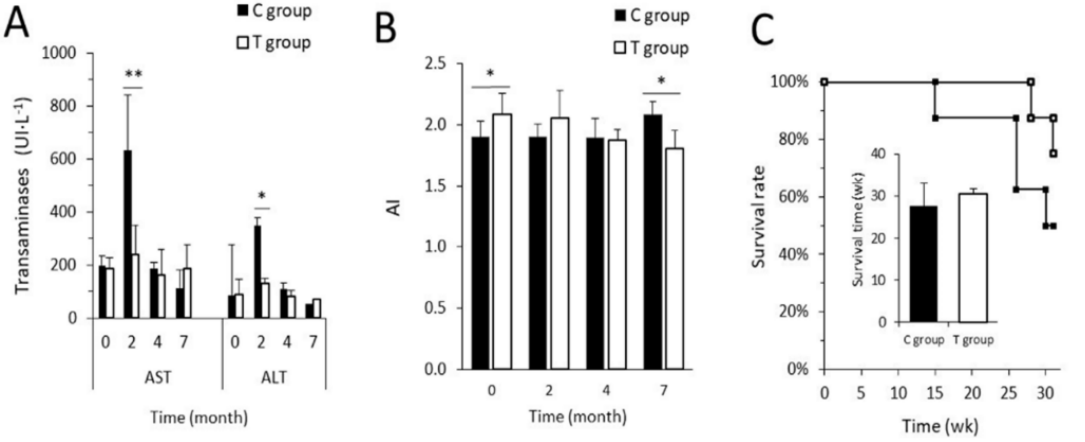

Figure 3. Transaminases Level, Atherogenic Index and Survival Rate in ZDF. (A) Transaminase levels. Statistical significant differences were observed in AST and ALT at 2 months of study, being that the C group clearly shown higher concentrations of both AST (632.1 ± 208.9 versus 238.9 ± 112.6 UI L^{-1} in C and T groups respectively; $p = 0.001$) and ALT (348.0 ± 192.2 versus 90.0 ± 54.6 UI L^{-1} in C and T groups respectively; $p = 0.019$). (B) Atherogenic index. Statistical significant differences were observed in basal (1.9 ± 0.1 versus 2.1 ± 0.1 in C and T groups respectively; $p = 0.004$) and end point values of AI (2.1 ± 0.2 versus 1.8 ± 0.1 in C and T groups respectively; $p = 0.015$). (C) Kaplan-Meier plot. The T group demonstrated a greater survival rate (87.5%) compared to the C group (50%). The survival time was higher in the T group (27.6 ± 5.6 versus 30.6 ± 1.1 weeks in C and T groups respectively, $p = 0.106$) but no significant differences between both groups were found. Data are represented as mean ± SD. * $p < 0.05$; ** $p < 0.01$.

3.4. The administration of FFP allows the maintenance of normal pancreatic activity

Blood insulin levels, measured as C-peptide cleavage secretion, were determined at two different time points (4 months and 7 months) in both groups. The results indicate that although a tendency to protection in the secretion of insulin was observed in the group treated with FFP, no significant differences were found between T group and C group at 4 and 7 months (p = 0.234 and p = 0.792, respectively) (**Figure 4A**). Besides, when looking to the ability to synthetize insulin, no significant differences were detected in the total area of insulin-positive cells of pancreatic tissue in both experimental groups (p = 0.114). With the aim of evaluating β-cells efficiency in insulin synthesis, we assessed the correspondence between the quantified positive β-cell number and C-peptide levels in serum at the end of the study. The results suggest a higher value of C-peptide levels/ β-cell in the T group although no significant differences were found (p = 0.114) (**Figure 4B**).

With respect to the Homeostatic Model Assessment (HOMA) no significant differences were observed in the HOMA-IR (p = 0.114). However, statistical significant differences were found in the HOMA-β (p = 0.029), remarking a better β-cell functionality in the group supplemented with the FFP (**Figure 4C** and **4D**).

3.5. FFP supplementation induces a decrease in intestinal glucose uptake

To test whether the hypoglycaemic actions of FFP supplementation could be related to a decrease in intestinal sugar uptake, we measured the ex vivo a-MG uptake in intestinal everted rings from animals after treatment. A significant decrease in intestinal a-MG uptake was observed in animals receiving FFP compared to the control group (p = 0.029) (**Figure 5**).

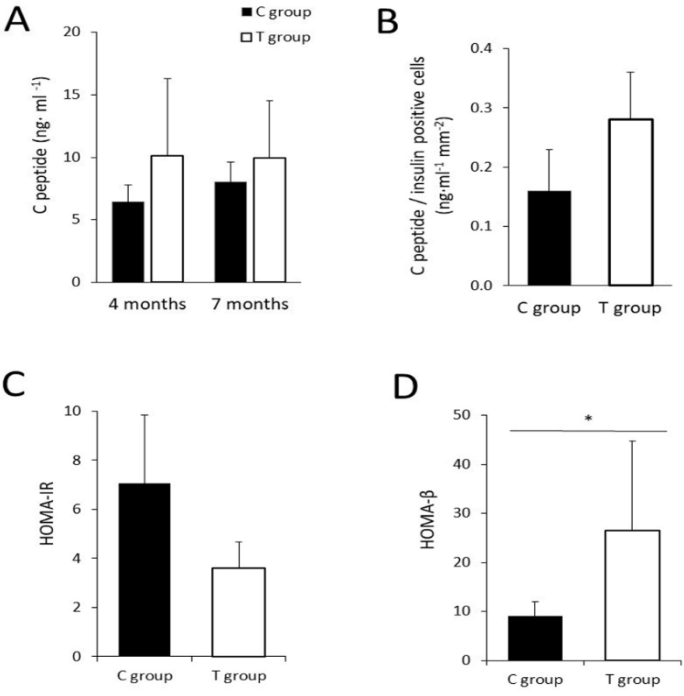

Figure 4. Pancreatic Function. (A) No statistical significant differences were found between serum C-peptide levels in C and T groups at 4 months (6.2 ± 1.7 versus 9.1 ± 6.6 ng ml^{-1} respectively; $p = 0.234$) and 7 months (7.6 ± 1.7 versus 8.5 ± 4.6 ng ml^{-1} respectively; $p = 0.792$). (B) When values of C-peptide/insulin positive cells were determined, a higher value was found in T group although statistical differences were not found (0.2 ± 0.1 versus 0.3 ± 0.1 ng ml^{-1} mm^{-2} in C and T groups respectively, $p = 0.114$). HOMA-IR (C) did not reflect differences among groups, however statistical significant differences were found in HOMA-β (D), between T and C groups (26.6 ± 18.2 versus 9.0 ± 2.9 respectively; $p = 0.029$); (n = 4 in C group and n = 4 in T group). Results are expressed as mean ± SD. * $p < 0.05$.

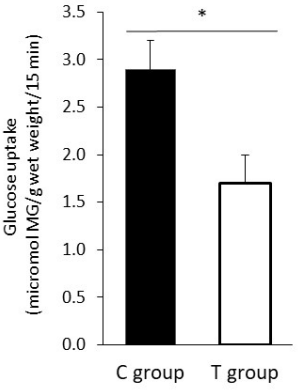

Figure 5. Intestinal Glucose Uptake. When intestinal glucose transport was determined, it was found that animals administered the FFP (T group) exhibited statistically significant lower marked glucose uptake compared to animals in C group (1.7 ± 0.7 versus 2.9 ± 0.6 micromol MG g wet weight^{-1} 15 min^{-1}; $p = 0.029$); (n = 4 in C group and n = 5 in T group). Results are expressed as mean ± SD. *$p < 0.05$.

3.6. The administration of the FFP altered the composition of faecal microbiota

At phylum level, Firmicutes, Proteobacteria, Bacteroidetes and Actinobacteria were the more predominant phyla present in the faecal samples of both groups (**Figure 6A**). After FFP supplementation, the percentage of Firmicutes was statistical significantly higher in the C group than in the T group (p = 0.017) and no statistical differences were found between groups in the Bacteroidetes phylum (**Figure 6B**), probably due to the variability observed. The ratio of Firmicutes to Bacteroidetes is a widely used indicator of the microbial composition, however in our study its value was comparable in both groups (1.23 versus 1.08 in C group and T group respectively). At the family level the more abundant taxa were *Lactobacillaceae* (4.1%), *Enterobacteriaceae* (3.6%), *Porphyromonadaceae* (3.6%), *Lachnospiraceae* (3.5%) and *Ruminococcaceae* (3.4%) (**Figure S4**). Statistical significant differences were only observed in *Streptococcaceae* (p = 0.046) and *Sutterellaceae* (p = 0.046) families. The *Streptococcaceae* family was higher in C group while the *Sutterellaceae* was higher in T group (**Figure 6C**).

The study of the total abundance of the genera revealed that the dominant bacteria genera were *Lactobacillus* (2.85%), *Clostridium* (2.26%), *Bifidobacterium* (2.26%), *Barnesiella* (2.22%) and *Bacteroides* (2.17%). From all 123 different identified genera, 4 and 19 were found exclusively in C or T groups respectively (**Tables S5** and **S6**). At genus level, the supplementation with the FFP enrich the abundance of Sutterella and Proteus, which were found more prominent in the T group (p < 0.001 and p = 0.032 respectively), while Anaerococcus and Streptococcus were more copious in control animals (p = 0.032 for both genera) (**Figure 6D**).

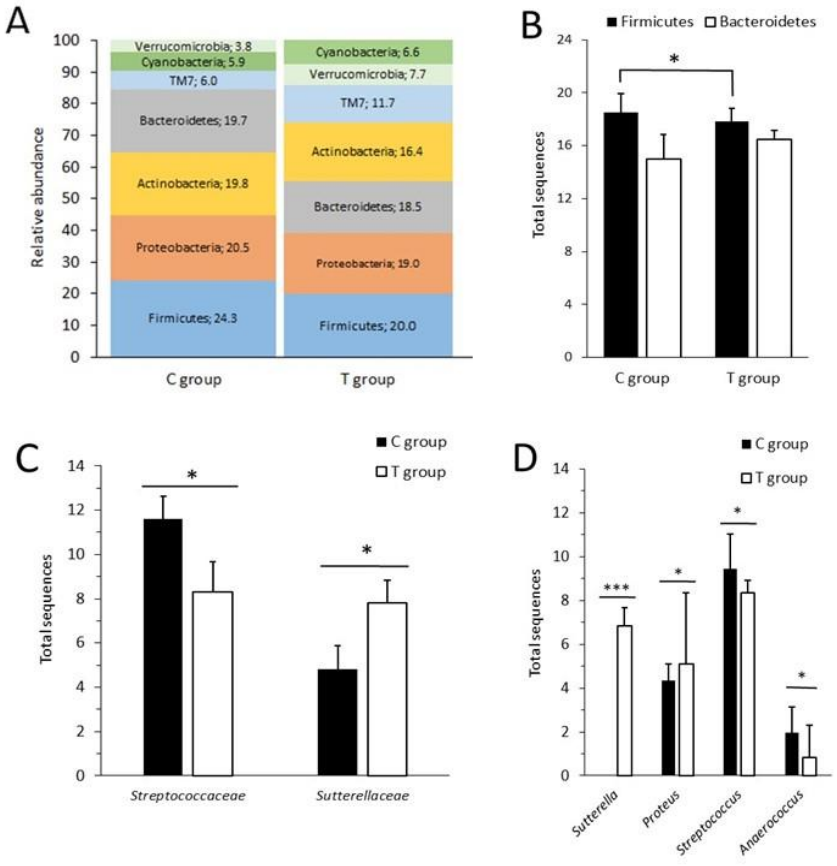

Figure 6. Faecal Microbiota Composition at Phylum and Family Level. (A) Relative abundance of phyla present in faecal samples in C and T groups. (B) Contributions of Firmicutes and Bacteroidetes. The abundance of Firmicutes was statistically significant higher in the C group than in the T group ($p = 0.017$) however no statistical significant differences were found in Firmicutes to Bacteroidetes ratio; (n = 3 in C group and n = 3 in T group). (C) Fecal microbiota composition at family level. *Streptococcaceae* contribution was significantly higher in the control rats than in the treated ones while *Sutterellaceae* was enriched in the treated animals; (n = 3 in C group and n = 3 in T group). (D) Widespread effect of the administration of the FFP on bacterial genera. Representation of statistically significant genera between groups at 6 months. *Suterella* and *Proteus* were found enriched in the T group whereas *Anaerococcus* and *Streptococcus* were more prominent in the C group; (n = 3 in C group and n = 3 in T group). * $p < 0.05$, *** $p < 0.01$.

A sum of 433 different bacterial species were identified in all the samples, of which 26 and 100 were exclusive of the C and T group respectively (**Table S5** and **S6**). A mutual core of 307 shared species was found in the two groups and among them 30 bacterial species significantly differed in the number of total sequences (**Figure 7**).

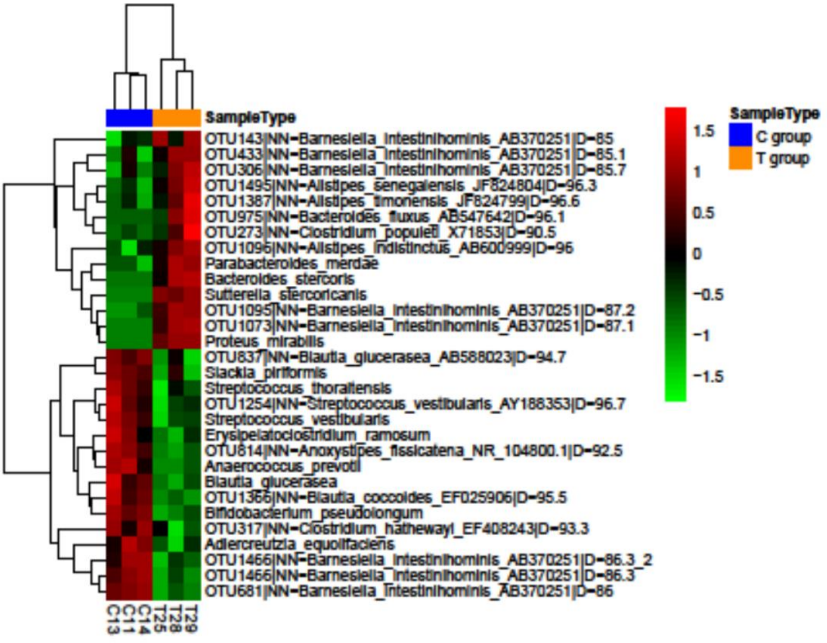

Figure 7. Hierarchical Clustering. Hierarchical clustering of differentially abundant species ($p < 0.05$) in C group and T group at 6 months; (n = 3 in C group and n = 3 in T group).

Diversity of the fecal microbiota were also analyzed. Alpha diversity indexes of bacterial community in ZDF rats are presented in **Table 1**. The alpha diversity was greater in T group than in C group, as samples in control group are perceived to have lower value in four of the five analyzed indexes. This may suggest that the administration of the FFP lead to an enrichment of the microbial diversity.

Table 1. Ecological Indexes of Alpha Diversity.

Sample	ACE	Chao1	Observed species	Shannon	Simpson
C group	2235.43 ± 314.85	2280.57 ± 322.74	1900.00 ± 298.08	4.99 ± 0.72	0.98 ± 0.01
T group	2837.85 ± 668.01	2868.51 ± 646.40	2510.67 ± 655.89	5.04 ± 0.76	0.98 ± 0.02

Values expressed as mean ± SD.

A non-metric multidimensional scaling analysis (NMDS) was performed in order to analyse the observed variability. The NMDS plot (**Figure 8)** showed that the distances between samples from the C group are shorter than those between samples in the T group. This means that C group presents a higher microbial homogeneity and so animals were more similar to each other than the treated animals.

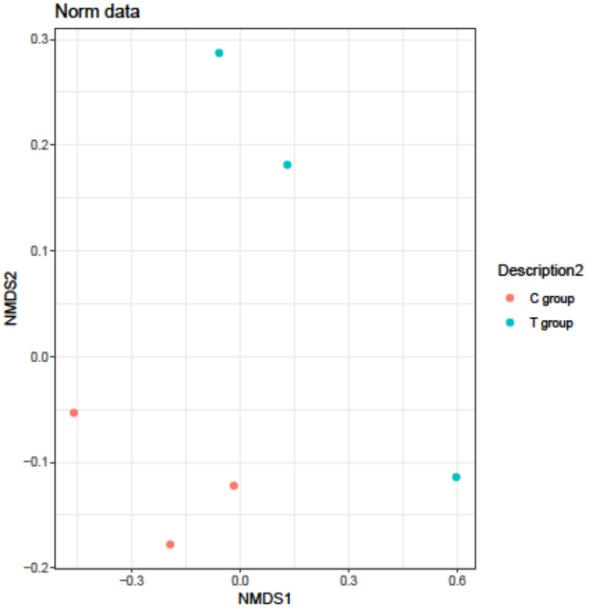

Figure 8. Non-Metric Multidimensional Scaling Analysis (NMDS). Representation of samples from C group and T group at 6 months; (n = 3 in C group and n = 3 in T group).

4. Discussion

Recent findings regarding T2D have led to new valuable knowledge on diabetic etiology and risk factors. Specifically, considerable interest exists in the role of the GM in the development and progression of diabetes. Several publications showed that diabetic individuals present a characteristic intestinal microbial community. This distinctive GM includes altered abundance of some bacteria taxa, for instance increased presence of *Lactobacillus* genera and opportunistic pathogens, along with differences in metabolic functionality, such as enhanced sulphate reduction and oxidative stress resistance [12]. Moreover, there is evidence that the GM influences the progression of the diabetes and its related complications [40] and its restoration resulted in clinical improvements according to experimental and clinical data. In particular, diet appears to play a key role and nutritional interventions have received attention for their ability to normalize the intestinal microbiota and thus improve health status [41]. In the present study a non-dairy fermented product was tested for its effectiveness in relieving the diabetic phenotype in the ZDF model. Among our experimental data, the markedly decreased glucose absorption in the treated group is probably the most striking result to emerge. Although extreme caution must be taken when extrapolating the results from ex vivo experiments, our result suggests that the total glucose which reach the systemic circulation was strongly attenuated with the FFP supplementation. This would support the lower BFG and GTT values observed in the T group.

We hypothesized that the FFP is able to improve the intestinal integrity and to decrease the load of glucose which reach the systemic environment, as suggested by previous authors [42]. A body of evidence has indicated an altered gut environment in diabetic people which includes a compromised tight-junction structure [43], a disrupted gut barrier [40], and impaired glucose transporters in the gut [42]. Taking it all into consideration, any approach that pursue to restore the gut health would eventually induce beneficial changes in the host.

A slightly higher value of C-peptide was also noticed in the supplemented group. The concentration of C-peptide represents an indirect estimation of β-cell activity as it leaks from proinsulin cleavage [44]. On the other hand, although the number of insulin positive

cells present in the pancreas of the C group was greater, we observed that β-cells were more efficient in the T group because a higher endogenous insulin production per β-cell was measured. Such findings indicate that the β-cell mass was best preserved in the T group. This is in good agreement with the HOMA-β score, and all together suggests a healthier pancreatic function in the T group. We believe that the treatment indirectly protected from organs dysfunction through a systemic effect, which together with the normalized values of glycemia observed in the T group in both BFG and GTT determinations could be responsible for the better pancreatic activity reported in this group.

Both the restoration of the glucose homeostasis and the protection of the pancreatic activity could be responsible for the improved wellness of the treated ZDF rats. A correct glucose metabolism can lessen alterations in cardio-metabolic parameters and result in a healthier lipid profile. Based on the physiological process of the T2D, also mimicked in the ZDF rat model, a deterioration in the lipid profile would be predicted along with the diabetes progression in both groups. However, our data reflected better values of TG, transaminases, and AI index in the T group. In this context, the observed results may suggest that the FFP helped to some extent to prevent diabetes-associated secondary alterations. Most of the analyzed parameters in the current research showed a tendency of a healthier status in the T group, and this trend was strongly manifested along the study by the greater survival observed in the supplemented group. As a result, the FFP-treated animals showed a prolonged survival in comparison with the control animals. It is also worth noting that the only animal that died in the group treated with FFP did so in week 28, while the first animal that did it in the control group was in week 15.

In addition to the mechanisms outlined above, another feasible explanation for the better glucose homeostasis in the T group is that the GM functional properties were affected by the FFP, and the ability to metabolize glucose was increased in some bacteria groups. Gut dysbiosis has been described in relation to glucose dyshomeostasis and emerging experimental and clinical evidence suggests that GM activity is modified in T2D [12,40,45,46]. Because of the key endocrine function exerted by the GM [47], restoring its

metabolic function is a valuable approach which proved to be successful in provoking significative changes [14]. In the metagenomic analysis of the fecal microbiome obtained from the ZDF rats performed in the present study we could identify some differences among groups' samples and some results did not confirm previous research on the topic. For instance, we did not find significant differences in Firmicutes to Bacteroidetes ratio. It is a relevant but controversial microbial marker. A reduced Bacteroidetes contribution or increased Firmicutes to Bacteroidetes ratio have been reported in obesity [48], and this parameter was found to be partially restored following weight loss [49] or prebiotic treatment [50]. On the contrary, other researchers have reported a significantly higher Firmicutes to Bacteroidetes ratio in lean than in overweighed or obese individuals [51]. In line with this observation, our results indicate a significant higher contribution of Firmicutes in the T group; however, our data did not reveal significant differences in the aforementioned ratio. Unlike other investigations carried out on the topic, we did not find a significant increase in *Bifidobacterium* levels. Based on the characteristics of the FFP, whose composition includes a high level of fermentable carbohydrates [52], a bifidogenic effect was expected in the T group due to the metabolites obtained during the fermentation process of FFP. This change is desired because this bacteria genera is a health marker [53]. However, our results indicate that the analyzed product or the experimental design could not induce a bifidogenic effect.

At the family level, *Streptococcaceae* was found significantly enriched in the control group, which matches with a previous type 1 diabetes (T1D) human trial [54]. On the contrary, *Sutterellaceae* family, which includes commensal species found in healthy human and animals [55], was more predominant in the treated group. At the genus level a significant enrichment was observed in *Sutterella* in the treated group. This finding does not corroborate previous results from earlier case-control studies in prediabetic subjects [56], experimental studies on type 1 diabetic animal models [57] and reports in other pathological conditions such as autism [58] and atopic dermatitis [59]. However, our finding matches to a previous investigation that associated barley consumption, a main component in FFP formulation, with an elevated abundance of *Sutterella* in the human GM [60]. *Proteus* genus was also enriched in the supplemented rats. Although there is no

evidence about the function of this genus in diabetes or obesity, some authors suggest its role in some pathological conditions [61,62]. In fact, one specie, *P. mirabilis*, was negatively correlated to health improvements [63,64], what suggests that a low abundance should be wanted. In contrast, our metagenomic analysis revealed that *P. mirabilis* was significantly enriched in the treated group. What is more, it was completely absent in control animal samples. We have no explanation for this striking result. Regarding the C group, *Anaerococcus* and *Streptococcus* genera were found more prominent. A previous report confirmed a lower relative abundance of the former genera in diabetic compared to healthy adults [65], contrary to our results. The latter genera, however, presented a greater relative abundance in prediabetic [56], type 2 diabetic [66], and type 1 diabetic individuals [67] compared to their healthy controls, what matches to our findings.

Among the 30 bacteria species statistically different between groups, eight *Barnesiella* spp. were identified and five of them were greater in the treated group. A previous experimental study reported an increased abundance of this genus in obese mice supplemented with prebiotics which experienced a better glucose tolerance and important metabolic improvements [68]. We also discovered that two *Blautia* spp. (*B. coccoides* and *B. glucerasea*) were more prominent in the control animals. This genus belongs to the family *Lachnospiraceae* and includes butyrate producing bacteria (BPB), a group which has been attributed many beneficial effects [69]. It was reported some positive correlations between *Blautia* spp. and microbial products such as long-chain triglycerides [70] and short chain fatty acids (SCFAs) [71], and parameters like BFG, insulin level, HOMA-IR, and weight loss [56,72]. A decreased abundance of *Blautia coccoides/Eubacterium rectale* was found in T1D children and was linked to intestinal disintegrity [73], while a randomized crossover study in healthy adults reported a reduced abundance of Blautia genus after prebiotic supplementation [74]. These results appear inconclusive. Our data also showed three *Alistipes* spp. enriched in the group supplemented with the fermented product. On the contrary, previous works reported its enrichment in T1D [67] and T2D individuals [12]. In the same way, our data revealed a lower abundance of two *Streptococcus* spp. (*S. thoraltensis* and *S. vestibularis*) in the group which exhibited a better glucose control (T group) and refute previous research [66].

The relative abundance of *Anaerococcus*, genus which includes many bacteria species which produce butyrate in experimental conditions [75], was significantly enriched in the treated group. Metagenome-wide association studies (MGWAS) on T2D humans revealed a compromised gut health with an abnormal abundance of BPB, thus an enrichment of the aforementioned bacteria group could, hypothetically, have led to improvements in the health status of the T group [12,46]. Regrettably, this study did not confirm previous research and the treatment with the FFP did not induce changes in well described BPB spp. such as *Akkermasia muciniphila*. *A. muciniphila* is a mucus producer belonging to the *Verrucomicrobiae* family which has been hypothesized to protect against T2D and obesity in animal studies [69,76]. Increased levels of *A. muciniphila* were correlated to improvements in health parameters in healthy [77,78] and HD-fed mice [76], and previous works in healthy mice showed that dietary manipulation such as supplementation with prebiotics could increase the abundance of intestinal *A. muciniphila* [79]. It seems that the nutritional properties of the FFP and/or the study design did not allow for a greater presence of *A. muciniphila* in the GM.

Regardless microbial populations, microbial metabolites are also of great importance and could be partly responsible for the improved glucose control and gut health found in the T group. Considerable interest exists in SCFAs such as acetate, propionate, and butyrate. They are lipid molecules known to mediate in inflammation [80], gut permeability [81], energy expenditure and metabolism [51,82], and an insulin-sensitizing [83] and antidiabetic effects [84,85] have also been described for them. Taking it into consideration, the promotion of their physiological levels can lead to an improved overall health, and may partly explain the favorable phenotype reported in the treated animals.

Since the FFP is rich in factors associated toa reduced food ingestion, such as fiber, microbial metabolites, and probiotic LABs [86–88], a decreased food intake was expected in the treated group. However, no considerable differences in eating behavior were observed between groups. This unexpected result is in good agreement with other previous studies with fermented dairy product in STZ-induced diabetic rats [25]. Similarly, BW was not significantly changed during the supplementation and was indeed more prominent on

the T group. Even though these results differ from an earlier in vivo study [89] and a crossover trial in prediabetic humans [18] that found a protection from weight gain for fermented products, they are consistent with previous finding in ZDF rats and other animal models of T2D [90,91]. Although no anti-obesity effect was observed for the FFP, it remains unknown whether it has some effect on body gain. May studies on another obesity rodent's model elucidate the impact of FFP on body mass regulation such as the ob/ob mouse model. The unexpected dramatic reduction in BW in the C group could be consequence of an acute failure of the pancreatic function. The decline in C-peptide levels in control rats suggest a low serum insulin level which, along with a compromised insulin signaling would promote lipolysis and favor fat mobilization from the tissue [92,93]. In much the same way, a FFP- mediated increase of SCFA levels in the T group, microbial metabolites known to downregulate lipolysis [94–96], could have prevented from the loss of weight in the treated ZDF rats. A previous report on *Monascus* fermented rice concluded that the differences in weight loss between control versus treated animals could be caused by a depletion in the lean mass in the former as results of diabetic complications [90]. In the present research, however, the nuclear magnetic resonance revealed the absence of discrepancies in body composition after FFP supplementation. It differs from previous results on fermented food in controlled intervention studies [18,97] and animals models [98] which evidenced a significant decrease in body weight and fat depots. Nevertheless, the absence of significant changes in body composition did not abstain from improvements in glucose homeostasis, what matches well with previous findings in murine models of obesity [99,100].

With reference to the microbial diversity, the results of the analysis strongly indicate that the FFP supplementation led to a greater diversity in the microbial communities which inhabit the gut environment of the ZDF rats. Another plausible explanation may be that the FFP abstained from the loss of diversity which consorts some clinical disorders such as obesity [48], however some controversy does exist with regards to T2D [12]. It is well- known that dietary factors, along with other external agents, have a great importance for the diversity and composition of the GM [101–103]. Previous works already studied changes in alpha-diversity with the consumption of diverse functional compounds. For instance, a meta-analysis compared different prebiotic treatments, and their efficacy in

increasing bacteria richness was found to be dependent on the fiber used and the diversity index calculated. The authors concluded that fiber interventions did not increase alpha-diversity [104]. They however suspected that a longer exposition would reveal some differences, what is in good agreement with our findings. Further, other authors reached the conclusion that the bigger the diversity of the GM, the bigger its resilience against external challenges [13]. This might consequently provide a healthier phenotype since a low bacteria richness was found to be present in illnesses and pathological conditions [103,105] and may this stability explain the better status reported for the treated group in our study. However, a compromised gut richness is not always present on T2D according to findings from a MGWAS with diabetic individuals [12] and bacterial diversity should not be the only focal point. Some authors call for a deeper approach and suggest that GM metabolic functionality, and not only its phylogenetic composition, could be an interesting target for future research and shed light on this point [106]. Then a more comprehensive approach is recommended.

This is not the first study reporting a hypoglycemic effect of probiotic bacteria or fermented products in animal models, however, there is a critical need for well-designed, controlled studies in humans to provide solid evidence of the suitability of fermented food for T2D management or prevention. Although the evidence from controlled trials in humans is limited and arises from small sample sizes [18,107], the number of work assessing the antidiabetic effects of fermented products in humans continues to grow [98,108]. Different fermented food products were investigated for their ability to exert antidiabetic effects and a wide variety of outcomes and levels of scientific evidence were reported [27]. On account of the fact that the study sample was small, and it was a preliminary attempt to test the FFP on an in vivo model, we strongly believe that a bigger sample size would have evidence more differences in the analyzed parameters. Nevertheless, previous supplementation with live bacteria in a different T2D murine model also failed to find significant differences in some metabolic markers and it did not abstain from relevant beneficial effects in pancreatic function and glucose homeostasis [109]. Nonetheless, the present research presents a valuable characteristic regarding its experimental design. In contrast to some reports in the literature in which the supplementation lasted for a few weeks [18,90,97],

we supplemented the rats for a longer time period (31 weeks). This prolonged exposition allowed us the examination of long-term responses.

Notwithstanding, as discussed above, we strongly believe that more work is needed to further understand how FFP works and thereafter, validate its potential effectiveness in diabetic patients. A future double-blind, placebo-controlled study with T2D individuals is being considered and would provide insight into the potential antidiabetic properties of FFP in humans. As not all the strains belonging to the same specie shares exactly the same beneficial effect [28,29], probably because of tiny differences in their physical and chemical properties [110], a full and comprehensive identification of the multi-species consortia of microorganisms presents in FFP, preferably up to strain level [111], would be of great important for it further characterization.

5. Conclusions

In summary, we demonstrated that the FFP was favorable on glucose metabolism and contributed to health maintenance, abstained from T2D harmful effects and improved overall life expectancy. Our study is in line with previous studies showing that modulation of GM can confer health benefits on T2D and OB. However, it is a fundamental issue to determine which component(s) present in the FFP is/are responsible for the observed beneficial effects. Research into solving this dilemma is already underway and we hope we could elucidate this issue. Future works on the topic would clearly be worth pursuing. Importantly, the undeniable disparities between experimental models and humans challenge the extrapolation of data from in vivo studies to humans, and a large well-controlled trial with an appropriate study design and statistical methods is needed to provide firm evidence of FFP's antidiabetic properties. These findings spotlight once again the role of microorganisms and gut function on the diabetic pathology and indicate that novel fermented products could be a powerful tool to protect against metabolic alterations. Nevertheless, very few publications are available in the literature that address the application of fermented products in diabetic humans and discordance within conclusions

makes difficult the elucidation of reliable markers. Our results open the possibility to explore the effectivity of innovative fermented food products in T2D, OB, and other non-communicable diseases in a near future.

~ Graphical abstract and Supplementary materials are available in **Annex, pages 261-270.**

6. References

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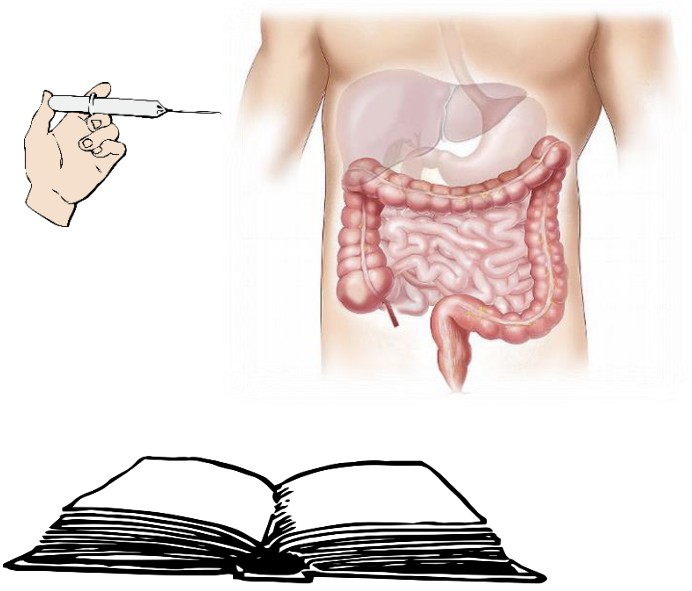
New Insights into Immunotherapy Strategies for Treating Autoimmune Diabetes.



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Abstract: Type 1 diabetes mellitus (T1D) is an autoimmune illness that affects millions of patients worldwide. The main characteristic of this disease is the destruction of pancreatic insulin-producing beta cells that occurs due to the aberrant activation of different immune effector cells. Currently, T1D is treated by lifelong administration of novel versions of insulin that have been developed recently; however, new approaches that could address the underlying mechanisms responsible for beta cell destruction have been extensively investigated. The strategies based on immunotherapies have recently been incorporated into a panel of existing treatments for T1D, in order to block T-cell responses against beta cell antigens that are very common during the onset and development of T1D. However, a complete preservation of beta cell mass as well as insulin independency is still elusive. As a result, there is no existing T1D targeted immunotherapy able to replace standard insulin administration. Presently, a number of novel therapy strategies are pursuing the goals of beta cell protection and normoglycemia. In the present review we explore the current state of immunotherapy in T1D by highlighting the most important studies in this field, and envision novel strategies that could be used to treat T1D in the future.

Keywords: diabetes; autoimmunity; insulin; immunotherapy and clinical trials

1. Introduction

Type 1 diabetes mellitus (T1D) is a pathology emerging from the selective elimination of pancreatic insulin-producing beta cells mediated by an autoimmune defect. Consequently, the main characteristic of this disease that occurs in its advanced stages is hyperglycemia. This form of diabetes accounts for approximately 5–10% of all diabetic patients. The prevalence of this pathology indicates that more than 500,000 children suffer from type 1 diabetes worldwide, mostly in North America and Europe [1]. However, the epidemiologic studies suggest that the incidence of T1D has increased markedly in recent years [2]. In 2017, the International Diabetes Federation (IDF; https://diabetesatlas.org) declared 132,600 newly diagnosed T1D cases worldwide.

1.1. Genetics if T1D

One of the main characteristics of T1D is the loss of beta cell tolerance, a process that involves different factors [3,4] including genetic associations with human leukocyte antigen haplotypes (HLA) and several beta cell-specific genes [5].

T1D is described as an inflammatory disease in which the infiltration of the pancreatic islets with a number of immune cell types (CD4+ and CD8+ T-cells, macrophages, dendritic cells (DC), and B cells) play a significant role [6]. The progression of islet infiltrates promotes beta cell elimination that ultimately results in the onset of diabetes.

While having some benefits, the transplantation of pancreas or pancreatic islets (Edmonton Protocol) [7] have had limited success due to the insufficient number of donors and the reactivation of the autoimmunity status despite immunosuppression protocols. Additionally, pancreas transplants have been demonstrated to be only partially successful [8].

1.2. Immunological Mechanisms Involved in T1D Pathogenesis

The progression of T1D can be divided into three critical stages [9,10]. At the "first stage", which may take place through a long period of time, individuals develop beta cell autoimmunity, identified by serum autoantibodies. The most frequent autoantibodies in T1D patients are those against GAD (GAD65), the tyrosine phosphatases IA-2 and IA-2, zinc transporter 8 (ZnT8), and insulin [11]. Those epitopes can induce the activation of CD4+ and CD8+ T-cells, which are the main mediators of beta cell destruction.

The presence of diabetes autoantibodies plays an important role in the identification of preclinical stages of T1D. The TrialNet TN01 has analyzed the importance of the autoantibodies markers for the detection of diabetes [12]. Five percent of the people screened through this study were found to present blood autoantibody. This study also determined that 95% of patients that progress to symptomatic T1D were autoantibody positive by the age of 5 years [13].

The identification of autoantibodies in the TEDDY (The Environmental Determinants of Diabetes in the Young) study showed a peak between 2 and 9 years of age [14]. Individuals that demonstrate the presence of at least two different autoantibodies have a significant chance of developing T1D [12,15].

Additionally, different HLA haplotypes were identified to be either protective or predisposing to diabetes development [16]. When autoreactive CD4+ and CD8+ T-cells begin to extinguish beta cells, the insulin levels start to decrease, which initiate the "second stage" of the T1D. At this stage, the main strategy for T1D treatment would be to suppress beta cell autoimmunity along with protection of the remaining beta cell mass. Different studies have demonstrated that at the time of diagnosis, which overlap with the second stage, there are still residual beta cells present (Clinical trial NCT01030861) [17]. Administration of immunosuppressive drugs in children with new onset of T1D can delay or reverse diabetes progression; however, immunosuppression can also result in organ toxicity. The diabetes progression resumes once the treatment is withdrawn [18].

The "third stage" of T1D occurs in long-term patients. At this stage, the main objective is to

ensure the functionality of the remaining beta cell. The studies have shown that following the disease onset there is a considerable reduction in C-peptide levels, a short polypeptide that connects insulin's chains in the proinsulin molecule and can be used as a surrogate of how much insulin is produced (Clinical trial NCT01030861) [17]. The maintenance of a high beta cell number could help in the control of hyperglycemia as well as to reduce the comorbidities of the disease.

It has been demonstrated that CD8+ and CD4+ T-cells, macrophages, and B cells are present in human cadaveric T1D pancreata [12,19]. However, the lack of insulitis in some T1D cadaveric pancreata samples underlines the heterogeneity of the disease [20] which could be one of the reasons why immunotherapies have not been fully effective in T1D patients.

Different immunotherapies have been proposed for all three stages of T1D. One approach involves the manipulation of the immune response, by using antibodies that target specific immune mediators. Another approach takes advantage of beta cell antigen-specific treatments. Interestingly, a treatment based on oral insulin administration demonstrated a delay in the diabetes onset in Non-Obese Diabetic (NOD) mice [21], an animal model which has been heavily used to study the progression and pathogenesis of T1D, and which we will describe in the next section.

1.3. Animal Models of T1D

Two different animal models have mostly been used in the field of T1D research: The NOD mouse and Bio-breeding (BB) rat. Both models exhibit the main symptoms of diabetes: Glycosuria, polyuria, weight loss, and islet of Langerhans-lymphocytic infiltration [22,23]. However, due to the implication of the T-cell compartment in the pathogenesis of T1D, the NOD model has been preferably used for the study of the diabetogenic T-cells development [24]. NOD mice show similar characteristics to human diabetes, summarized in **Table 1**.

NOD mice were originally generated in the Cataract Shionogi (CTS) strain [22]. Cell infiltration in the pancreas of NOD mice can be observed at as early as 3 weeks of age. This

process includes the recruitment of different innate immune cells into the islets of Langerhans including macrophages and neutrophils, prior to the infiltration of the lymphocytes [25,26]. Although the presence of autoreactive T-cells is initially low, their numbers gradually increase, due to the recognition of certain diabetes-specific autoantigens and become activated, initiating the elimination of insulin-producing beta cells. Despite the focus of T1D research on T-cell-mediated beta-cell destruction, there are studies showing that B cells also play an important role in the diabetes onset [27].

The NOD mouse model has provided valuable information regarding the role of the immune cells in diabetes development. Furthermore, NOD mice have provided a unique research tool in order to explore immunotherapy treatments (i.e., CTLA4-Ig, anti-CD40 antibodies, and IL- 4 or IL-10 treatment), as has been exhaustively reviewed by Shoda et al. [28]. However, most of the immune-interventions that have shown promise in the NOD mouse model failed to demonstrate similar impact on human disease. For this reason, the attempts to humanize NOD mice [29] might facilitate the research that would eventually translate into successful immunotherapy clinical trials.

Additionally, some external factors also play an important role in T1D development. The studies in monozygotic twins have demonstrated a lack of concordance suggesting the importance of environmental factors in the T1D progress. Many of those factors have been involved in modifying diabetes susceptibility in NOD mice, including changes in the gut microbiota [30–32]. The interaction of innate immune components with the gut microbiota represents a hot topic in the field of T1D research. We will deepen this aspect in Section 6.2.

Apart from the previously mentioned mouse models, another useful model is the DO11.10xRIPmOVA (DORmO) mouse model, where RIPmOva animals (mice that express membrane-bound OVA in thymus and pancreas) are crossed with DO11 animals expressing an OVA-specific MHC-II TCR. Somehow surprisingly, these double-transgenic animals generate large numbers of islet specific functional Treg cells (see Section 2.3), but spontaneously develop T1Dby week 20. Therefore, the DORmO model is uniquely suited to study Treg role in T1D initiation/progression [33,34]

Table 1. Autoimmune Diabetes Developed by NOD Mouse Compared to Human T1D

	NOD	Human
Age at onset	> 10 weeks	>6 months-late adolescence
Genetic Susceptibility	MHC most important	HLA most important
Autoantigens	Insulin, GAD, IA-2, IA-2b, ZnT8, IGRP, Chromogranin A	Insulin, GAD, IA-2, IA-2b, ZnT8, IGRP, IAPP, HSP60, Carboxypeptidase H
Insulitis	DCs, Macrophages, B cells, NK cells, CD4 & CD8 T	DCs, Macrophages, B cells, NK cells, CD4 & CD8 T
Ketoacidosis	Mild	Severe
Gender effect	Female predominantly affected	Males and females almost equally affected

1.4. Present State of T1D Immunotherapy

Current strategies for T1D immunotherapies could be classified as antigen-independent and antigen-dependent. Antigen-independent (non-antigen specific) interventions include: drugs that induce immunosuppression, antibody-based therapies that allow the depletion of polyclonal B or T cells [35], cytokine-based strategies [36], and the increase of tolerogenic DC [37], and polyclonal Treg cell numbers [34].

Antigen-dependent (antigen-specific) strategies involve the use of beta cell-derived autoantigen-based vaccines, adoptive transfer strategies and specific abrogation of autoreactive T-cell clone by targeting antigen presentation mechanisms [38,39].

The combination of different interventions based in immunotherapy treatments is considered the most effective strategy due to the complexity of T1D [40].

In the next sections, we will analyze the strategies of immunotherapy that are currently used for treatment and prevention of T1D (summarized in **Table 2** and **Figure 1**).

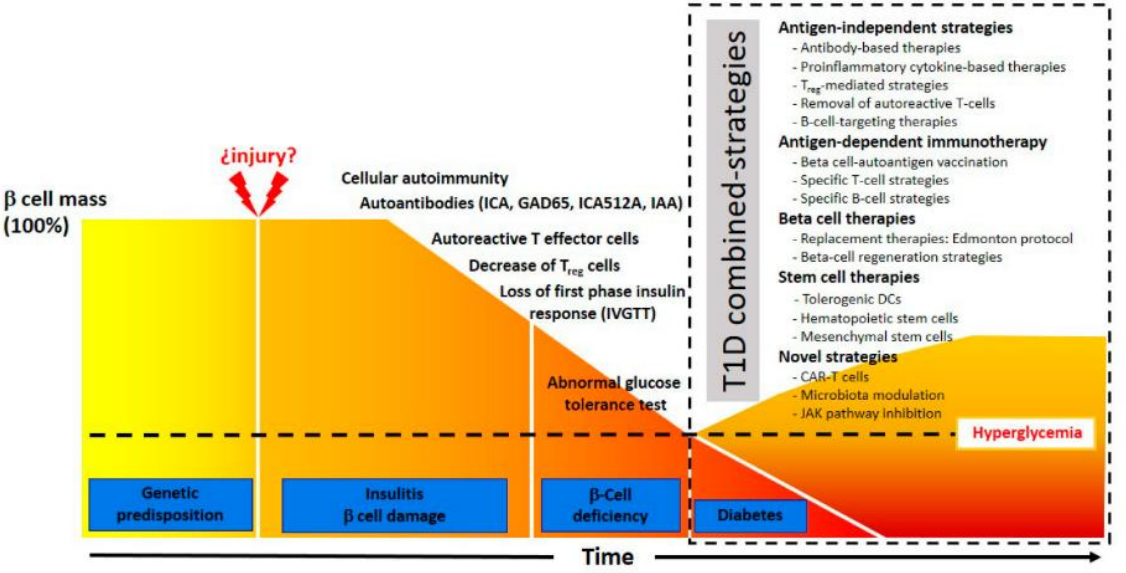

Figure 1. Progression of T1D and Combined-Strategies for T1D Treatment.

Table 2. Strategies for the Treatment of T1D.

Antigen-Independent Strategies	References
Antibody-based therapies	
Anti-CTLA-4	Clinical trial NCT01773707
Anti-CD3	Clinical trial NCT01030861
Anti-CD2	[18]
Anti-thymocyte globulin (ATG)	[35]
Proinflammatory citokine-based therapies	
IL-1a/IL-1b	[41]
TNF	[42]
Nicotinamide	[43]
IL-12/23	Clinical trial NCT02117765
IL-6	Clinical trial NCT02293837
Treg -mediated strategies	
Treg suppression	[44,45]
Removal of autoreactive T-cells	
Anti-CD3	[46,47]
B-cell-targeting therapies	
Anti-CD2	[48]
Antigen-dependent immunotherapy	
Beta cell-autoantigen vaccination	
GAD	[49]
Specific T-cell strategies	
Tolerized T effector cells	[50]
Specific B-cell strategies	
Depleting insulin-reactive B cells	[51]
Beta cell therapies	
Replacement therapies	
Edmonton protocol	[7]
Beta-cell regeneration strategies	
Gastrin + GLP-1	[52,53]
Stem cell therapy strategies	
Tolerogenic DCs (tDCs)	
Autologous tDCs	[54,55]
Combination tDC + Tregs	[56,57]
Hematopoietic stem cells (HSC)	
Autologous myeloablative HSC transplantation	[58]
Autologous non-myeloablative HSC transplantation	[59]
Mesenchymal stem cells (MSC)	
Autologous MSCs	[60,61]
Allogeneic adipose-derived MSCs	Clinical trial NCT02940418
Umbilical cord blood MSCs (UC-MSCs)	[62]

2. Antigen-Independent Strategies

2.1. Antibody-Based Therapies

The activation of T-cells is controlled by various costimulatory pathways which could be positive or negative. For example, signaling through CTLA4 induces an anergic state in naïve T-cells, and therefore Abatacept, a fusion protein composed of the Fc portion of human IgG1 fused to the extracellular domain of the CTLA4, is used for treatment of rheumatoid arthritis [63]. In a recent clinical trial, abatacept has demonstrated potential against T1D by delaying C-peptide exhaustion in T1D patients [41]. The clinical trial TrialNet is analyzing the benefits of abatacept in the delay of early T1D onset (Clinical trial NCT01773707; www.clinicaltrials.gov).

Anti-CD3 monoclonal antibodies that target CD3/T-cell receptor (TCR) complex, blocking the union of CD3 with TCR and rendering an anergic state of the T-cells have also been tested in T1D patients. Teplizumab and otelixizumab, two of the main clinically approved anti-CD3 antibodies, have demonstrated some efficacy in T1D patients [42]. Teplizumab treatment induces a delay in C-peptide decay in treated T1D patients. In this study AbATE, 2 week-teplizumab treatment resulted in C-peptide preservation [17] [Clinical trial NCT01030861; www.clinicaltrials.gov]. The main results from this clinical trial are expected to be released at the end of 2019.

Among the selective ablation of T effector cells, the elimination of memory T-cells would also be necessary in order to obtain long-lasting results. This could be achieved by inhibiting CD2 signaling. The anti-CD2 fusion protein Alefacept efficiently blocks T-cell activation inducing apoptosis of both memory and effector T lymphocytes. Unfortunately, only a modest trend towards preserving C-peptide levels was achieved when this hypothesis was tested during the clinical trial T1DAL, which included patients at late stage of T1D [18].

Lastly, anti-thymocyte globulin (ATG) have been described to be able to deplete activated T-cells. ATG used in low doses in combination with granulocyte colony-stimulating factor (G- CSF) showed that it is safe and can induce protection of beta cell mass [43]. Later clinical

trials demonstrated that G-CSF by itself did not provide any additional benefits [64].

2.2. Proinflammatory Cytokine-Based Treatments

The role of inflammation and proinflammatory cytokines have been long known to have a role in T1D development [65]. Inhibition of expression of those molecules can induce important changes in pancreatic beta cells [44]. Such strategy was clinically used for treatment of other autoimmune diseases [65].

Interleukin (IL)-1α and IL-1β are important immunomodulators expressed by monocytes that can induce a toxicity on beta cells [45]. Anti-IL-1 administration for rheumatoid arthritis has been proven to be well tolerated in patients [46]. IL-1 is also involved in T1D progression by activating T helper cells, and improving the number of circulating memory T-cells [47]. A clinical trial performed on T1D patients suggested that IL-1 inhibition could induce a preservation of pancreatic beta cells [66].

Another cytokine that plays an important role as an intermediary molecule in autoimmune diseases is tumor necrosis factor α (TNF-α). Therefore, the blockade of TNF- has been tested as a treatment of autoimmunity. Regarding T1D, patients that were treated with Etanercept (recombinant TNF-α receptor–IgG fusion protein) had improved preservation of beta cell mass (assessed by the C-peptide levels) and decreased glycated hemoglobin levels [48].

The ability of nicotinamide alone or in combination with vitamin E to preserve functionality of remaining beta cells has also been tested. Both treatments proved to be effective in retaining the basal secretion of C-peptide [67].

The IL-12/23 cytokine pathway, which is involved in the induction of inflammatory cytokines and pathogenic T-cell activation, was also considered as a potential therapy for T1D therapy. The application of Ustekinumab (IL-12/23 blocking molecule) has been tested in patients with T1D (UST1D clinical trial) (Clinical trial NCT02117765; www.clinicaltrials.gov).

Overexpression of IL-6 was noticed in a subset of T1D patients [68]. As a result, anti-IL-6 therapy, which is also tested in managing arthritis and systemic juvenile idiopathic arthritis [69], was initiated. Currently, the clinical trial EXTEND (Clinical trial NCT02293837; www.clinicaltrials.gov) is examining whether the blockade of IL-6 signaling (tocilizumab, an anti-IL-6 receptor antibody)) can induce a protection of beta cell function in T1D patients (ages 6 to 17 years) is ongoing.

Taking all the data together, cytokine inhibition is emerging as a viable supplementary approach in order to achieve durable therapeutic efficacy of T1D treatment.

2.3. Treg-Mediated Strategies

Tregs have also been involved in the pathophysiology of T1D [70]. Bluestone and colleagues examined the role of expanded autologous polyclonal Tregs in the treatment of T1D patients [70]. In this clinical trial, autologous Treg infusions were safe, but did not modify the course of the disease. Other clinical trials have also evaluated the effects of low doses of IL-2 on Treg activity [71]. Although IL-2 was able to increase the total number of Treg, this did not result in better glycemic control.

Intriguingly, recently published data has shown that blocking extracellular deposits of the polysaccharide hyaluronan (HA) (frequently observed in T1D patients [72]) reduced diabetes in two different mouse models by significantly enhancing the percentage of Treg in pancreatic islets and preventing further β cell destruction [34].

2.4. Removal of Autoreactive T-cells

Targeted depletion of autoreactive T-cells in T1D patients is an approach with great potential, as it aims to eliminate effector T-cells responsible for the destruction of pancreatic beta cells. Treatment of NOD mice with anti-CD3 antibodies was shown to induce anergy in T-cells [49]. Additionally, elevated high counts of Treg cells were observed in patients administered with anti-CD3. A clinical trial performed with T1D patients

showed reduced insulin requirements after treatment with the anti-CD3 antibody [42,73]. No severe adverse events were observed, and even mild sides effects were rarely reported. These results suggest that anti-CD3 antibody treatment can be considered as a potential treatment for T1D [49].

2.5. B-Cell-Targeting Therapies

Since, B cells were implicated to take part in beta cell destruction through autoantibodies production, targeting of B cells in T1D settings has also been studied. The elimination of B cells in NOD mice prevented the accumulation of auto-antibodies, thus averting diabetes onset [74]. T1D patients treated with anti-CD20 antibodies showed higher C-peptide levels and lower insulin dependency when compared to the placebo group. However, this strategy does not seem to completely prevent C-peptide decay [50,75].

3. Antigen-Dependent Immunotheraphy

In contrast to antigen-independent strategies, autoantigen-targeting treatments of T1D could modulate specifically T1D-related autoimmunity while preserving the normal immune homeostasis. The main objectives of antigen-specific therapies is to induce tolerance of autoreactive T effector cells and expansion of autoantigen-specific Treg cells [38,39].

3.1. Beta Cell-Autoantigen Vaccination

The exposure of specific antigens to naïve T-cells could induce immune tolerance to that antigen. According to current knowledge of T1D progression, we can hypothesize that antigens derived from beta cells that are applied in a non-inflammatory context might modulate autoreactive T-cells, resulting in beta cell preservation [76]. This paradigm has led to developing novel vaccination strategies to achieve the induction of T-cell tolerance

against specific autoantigens. The well-known T cell epitopes against insulin and glutamic acid decarboxylase (GAD) have been extensively studied [51], demonstrating that C19-A2 proinsulin peptide could modulate autoreactive CD4+ T-cells in patients with specific class II allele [77]. The administration of this peptide in recently diagnosed T1D patients resulted in the exhibition of higher C-peptide levels without symptoms of systemic or local hypersensibility [78].

Additionally, another T1D autoantigen, GAD65, was targeted in NOD mice in order to reduce the number of GAD65-specific T effector cells [79]. Normoglycemia was achieved in 70% of NOD mice, and in 80% of them normoglycemia persisted in long-term post-antigen administration.

Despite the successful results observed with vaccination strategies in NOD mice, the dissimilarities in autoantigens between human and mice and the heterogeneity of T1D in humans makes this strategy not very suitable for clinical application [52].

3.2. Specific T-Cell Strategies

The dysfunctional imbalance of Treg to T effector cells is an important factor determining the onset of T1D [80]. CD8+ T-cell activation is a process mediated by the presentation of specific epitopes from professional antigen-presenting cells (APCs) as DCs appear to be the principal APCs for the CD8+ T-cell [81]. The process depends on CD4+ T-cells' interaction that induce the activation of specific subsets of CD8+ T-cells which in turn is responsible for initiating islets' beta cell destruction [53].

The process of achieving self-tolerant T effector cells could be through use of either the whole antigen or specific peptides. However, success intolerization of T effector cells depends on different factors, especially the identification of the autoantigen that drives this process. In order to prevent beta cell destruction, the most relevant T effector clones have to be deleted.

3.3. Specific B-Cell Strategies

The strategy based on the abrogation of non-specific B cells has not been very effective. However, inhibition of specific autoantigen B cells by depletion of insulin-reactive B cells, is a promising alternative [82]. Insulin-specific B cells elude the immune control in NOD mice responding to insulin by increasing the expression of costimulatory molecules during the crosspriming of effector T-cells.

4. Beta Cell Therapies

4.1. Replacement Therapies: Edmonton Protocol

The Edmonton protocol has shown the value of islet transplantation in addressing insulin regulation in T1D patients [7]. According to this protocol, pancreatic islets obtained from cadaveric donors are infused into immunosuppressed T1D patients.

Trials conducted before 1990 using single islet infusions were partially successful, as they resulted in lower insulin needs and higher C-peptide levels; however, no additional steps to increase the net islet mass of the transplant had been taken in any of those trials [54].

Islet transplantation protocols became a promising therapy for type 1 diabetes thanks to the introduction of the Edmonton Protocol in 2000. Today this method is the only therapy that can reach glycemic control without the administration of insulin [55]. Transplantation of pancreatic islets has several advantages over the transplantation of a complete pancreas, since it involves only a minor surgical procedure with low morbidity and mortality, and a significantly lower cost. The main advantage of islet transplantation protocols over conventional insulin therapy is that transplanted islets are more efficient in maintaining normal blood glucose levels without producing excess insulin that could lead to episodes of hypoglycemia.

Modifications of the Edmonton Protocol based on a new immunosuppression regimen have prevented the use of corticosteroids, allowing the application of a unique combination therapy based on anti-interleukin-2 receptor antibodies along with the

immunosuppressant drugs sirolimus and tacrolimus. The main advantage of this combination treatment is low beta cells toxicity. Islet transplantation has shown some success regarding insulin independence both in the short and long term [55,83] as much of the variability in the results obtained with the Edmonton Protocol is associated with factors related to both the organ donor and the recipient.

Although the benefits of the islet transplantation protocol are unquestionable, among the concerns for standardization of this strategy are the large number of islets that have to be transplanted and the adverse effects derived from the immunosuppression regimen. The first problem could be addressed by using stem cells that, under the adequate differentiation protocol, are able to differentiate into glucose sensitive insulin-producing cells (see Section 5.3).

4.2. Beta-Cell Regeneration Strategies

Gastrin and GLP-1 have a synergistic effect in inducing the regeneration and differentiation of beta cells [56,57]. In the NOD mouse model, the addition of both molecules resulted in increasing of beta-cell mass [58]. In addition, the combination therapy with DPP-4 inhibitors, (to increase GLP-1 levels), and proton pump inhibitors (PPIs; to increase gastrin levels), increased C-peptide levels and insulin secretion, and restored the normoglycemia in NOD mice [56]. In humans, the study REPAIR-T1D analyzed the effect of one-year similar treatment using a combination of sitagliptin (DPP-4 inhibitor) plus lansoprazole (PPIs inhibitor) in T1D patients [60]. However, no differences in C-peptide levels were observed between treated vs. placebo groups [60]. The authors claim that the increase in gastrin concentrations and GLP-1 were low, resulting in non-efficient treatment. Further clinical trials will be required in order to determine the role of gastrin and GLP-1 combination therapy.

5. Stem Cell Therapy Strategies

5.1. Tolerogenic DCs

Although various cell types have been studied as potential targets for T1D treatment, dendritic cells attracted special interest. However, clinical trials in which T1D patients received autologous DCs showed limited results. In these clinical trials, DCs were infused via abdominal intradermal injections every 2 weeks [61]. Although the treatment was well tolerated, no significant differences on glycaemia were observed.

Previous studies demonstrated, that dendritic cells, alone or via different effector cells, such as Tregs and B-regulatory cells (Breg), could play an important role in the activation status of autoreactive CD8+ cytotoxic T-cells (CTL) as well as influence the balance between T-helper cells (Th1 and Th2) and effector cell populations [59]. Tolerogenic DC (tDCs) populations have been used in different clinical trials for treatment of autoimmune diseases, including T1D [61,84]. The results of those studies suggested that tDCs remain at the administration site promoting the generation of a lymphoid stroma tissue which in turn allows the increase of FoxP3+ Tregs [85].

The synergistic inter-relationship of tDCs and Tregs allows them to generate a very powerful tolerogenic state. Co-administration of tDC and Tregs, would allow stabilization of Foxp3 expression and would elevate the levels of IL-10, TGF-β and retinoic acid by tDCs [86,87]. The tolerogenic state of the tDC would be increased via cell–cell interactions or through paracrine mechanisms. This combination strategy may change the paradigm of how autoimmune diseases are being treated, addressing the disproportion of the immune effectors generated during the disease-onset.

5.2. Hematopoietic Stem Cells (HSC)

Although immune dysfunctions linked to T1D are complex, Voltarelli and colleagues published an innovative research, where newly diagnosed T1D patients enrolled in a phase 1/2 clinical trial received immunosuppression treatment together with the infusion

of autologous HSCs. The results obtained were promising; almost all patients did not require insulin injections for 6 months as their C-peptide levels stayed stable and the anti-GAD auto-antibodies levels were diminished [88].

In two recent prospective non-randomized trials, most patients showed no need for insulin administration after HSC transplantation [89,90]. The results of those studies showed that even 4 years post-transplantation, the C-peptide levels were still significantly higher than pre-transplant ones [89].

Recently, the results from a study using autologous non-myeloablative HSC transplantation were published [62]. Fifty-nine percent of the patients included in this clinical trial did not require insulin administration while 32% remained insulin-independent for at least 4 years [62].

Most of the patients included in the autologous HSC-transplantation clinical trials presented limited side effects. Only one clinical trial declared a patient death due to Pseudomonas aeruginosa sepsis [89].

Although the adverse effects related to immunosuppression protocol limit this alternative treatment, the administration of autologous HSC remains an exciting way forward in the task to find a cure for T1D.

5.3. Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) are stromal stem cells that play important roles in tissue repair and regeneration [91]. MSCs express specific antigen biomarkers (MHC I, CD90, CD105, and CD73) that enable their identification by flow cytometry techniques. MSCs have proven to be very promising in regenerative medicine thanks to their ability to give rise to different cell types, such as adipocytes, chondrocytes, and osteoblasts, making it possible to replace damaged tissues. [92]. In addition, MSC can be recruited from other injured tissues, such as ischemic heart or pancreas [92,93]. For this reason, MSCs are representing a new approach that will help the promotion of the integration of stem cell transplants in regenerative medicine protocols [94].

MSCs have been used to treat T1D patients and showed promising results in maintaining blood C-peptide levels [95]. However, no differences were observed for insulin requirements when compared with the non-treated group during the study.

The biological properties of MSCs regarding their potential to control aberrant immune response were demonstrated in NOD mouse model [96,97]. In Uppsala University Hospital's sponsored clinical trial, in which T1D patients were transplanted with autologous MSCs, treated patients exhibited a better maintenance of C-peptide levels [96]. Umbilical cord blood MSCs (UC-MSCs) were also tested in combination with autologous mononuclear cells derived from bone marrow (aBM-MNC) in another clinical trial. The results of this study showed that the infusion of aBM-MNC induces a 30% reduction of insulin requirements [98]. Nowadays, many trials are trying to test the use of MSCs from different sources for the treatment of T1D, including the use of allogeneic MSCs derived from adipose tissue (NCT02940418 and NCT02138331).

To date, the use of immunoregulatory MSCs is a very promising topic in the T1D stem cells field. The combination of MSCs with other immunotherapies would offer a novel strategy for the treatment of T1D patients.

6. Novel Strategies

6.1. CAR-T-Cell Therapy

6.1.1. Introduction

In the recent years, an immunotherapy using engineered T-cells expressing chimeric antigen receptors (CARs) specific against CD19 emerged as a major breakthrough in cancer therapy of CD19+ B-cell leukemia [99]. CARs are complex molecules composed of several components, the most common being: (1) An antigen-specific recognition domain, usually a single chain variable region (scFv) from a monoclonal antibody; (2) a hinge region, based on the Fc portion of human immunoglobulin (IgG1 or IgG4), or originating from the hinge domains of CD8a or CD28; (3) a transmembrane domain; and (4) an intracellular tyrosine-

based signaling domain [100]. The signaling domain is the engine of the receptor. Its most common component is the intracellular portion of CD3ζ, which is the main signaling chain of CD3 T-cell receptor (TCR) complex. The biggest advantage of CAR-T-cells is that the receptor's interaction with its antigen is independent from major histocompatibility complex (MHC) but it still activates the same TCR's and costimulatory intracellular signaling cascades necessary for T cell activation and expansion.

6.1.2. CAR-T-Cells and T1D

Based on the studies with CARs in cancer and increased interest of Tregs as a potential tool for T1D therapy (see Section 2.3). It is only logical to hypothesize that armoring Tregs with cell-specific CARs would improve Tregs' migration into the pancreas and pancreatic lymph node, thus protecting islet cells from autoimmune destruction. A number of recent studies suggests that there is big potential for CAR-Tregs therapy in multiple autoimmune or allograft rejection model systems [101–106]. Fransson and colleagues described an interesting approach for CAR-Tregs use in the EAE mouse model [105]. In their study, CD4+ T-cells were engineered to express both a CAR specific against myelin oligodendrocyte glycoprotein (MOG35-55) and a murine Foxp3 gene to drive Treg differentiation, separated by a 2A peptide sequence. Intranasal administration of CAR-Tregs resulted in a successful delivery to the CNS, an efficient suppression of the ongoing inflammation and complete recovery from disease symptoms. Other studies propose the use of CAR-Tregs in transplant rejection by generating HLA-A2-specific CAR-Tregs that were isolated from the host [102,104]. These HLA-A2-CAR-Tregs retained high expression of Foxp3, LAP, GARP, and CTLA-4, and maintained their suppression function in vitro without a significant cytolytic activity. Even though there is still necessity to confirm the stability of Treg phenotype, purity, and long term survival after the transfer, this approach is very promising for treating and prevention of transplant rejection by inducing graft-specific tolerance.

CAR-Tregs were also studied in Hemophilia A, where genetic mutations in F8 gene result in either reduced levels or altered functionality of the blood-clotting protein, Factor VIII (FVIII). In patients with severe hemophilia (no circulating FVIII can be detected), there is a

high probability for developing adverse immune reactions to the exogenously administered FVIII protein. Remarkably, administering FVIII-specific human CAR-Tregs suppressed antibody production in vitro and in vivo in a mouse hemophilia A model. However since FVIII is a soluble protein, the mechanism of this suppression is not entirely clear [101,107].

Hansen's group study was an additional proof of concept that CAR-Tregs are a prospective therapy strategy for multiple autoimmune conditions [106]. The authors generated CAR against carcinoembryonic antigen (CEA), a glycoprotein presented on lung adenoepithelia, and then adoptively transferred Tregs expressing this construct in an experimentally induced allergic asthma mouse model. The CAR-Tregs accumulated in the lungs and nearby lymph nodes, reducing airway hyper-reactivity, inflammation, mucus production, and eosinophilia.

6.1.3. Challenges

Despite the great potential of CAR-Tregs therapies, there is still no clear strategy on how to use this exciting technology for the treatment of T1D. The biggest challenge is the lack of cell-specific antibodies that can be harnessed to generate islet-protective CAR-Tregs. One possible approach to overcome this problem is to use human islet-specific TCR gene transfer to polyclonal human Tregs. A recent study where polyclonal Tregs were transduced with TCR chains derived from two human islet-specific CD4+ clones showed an improved antigen-specific suppression of these cells and increased potency when compared to polyclonal Tregs [108]. However, such islet-specific Tregs were less responsive to their cognate antigen in comparison to T-cells expressing virus-specific TCRs suggesting that further optimization and/or identifying better TCR clones is still needed.

A new study demonstrated that insulin-specific CAR-Tregs were functional, suppressive and surviving in vivo even though they were not able to prevent spontaneous diabetes in NOD mice [109]. This is not a surprise considering the fact that insulin is a soluble antigen that is present throughout the body and its concentrations fluctuate. Moreover, such a

strategy would not be very efficient in patients with T1D where endogenous insulin levels are very low and the daily insulin injections would disturb the normal insulin concentration gradient that might drive the insulin-specific CAR-Tregs into the pancreas.

Therefore, the discovery and study of new cell-specific molecules that could provide proper targeting of CAR-Tregs is needed. While there are some promising molecules such as DPP6 [110]), FXYD2 a [111], and NTPDase3 [112], all of those would require additional studies confirming their specificity, as well as isolating appropriate monoclonal antibodies that would recognize human cells in vivo before developing a CAR construct for T1D therapy.

6.1.4. Summary

In summary, despite the advances in the field of CAR-Tregs therapies and their great potential to be applied for autoimmune disorders, there is still a lack of an efficient system as well as of appropriate surface β cell-specific markers that would allow the generation of effective auto Ag-specific Tregs that could be used for cell-based therapies in T1D.

6.2. Microbiota Modulation

6.2.1. Introduction

The microbiota refers to a complex ecosystem of bacteria and viruses, among other microorganisms that inhabits our body, especially the digestive tract. This community greatly exceeds the amount of eukaryotic cells that form the human body and their collective genome, named microbiome, is considerably larger than the human genome. On account of the mutualistic relationship between the host and its gut microbes, the imbalance of the latter, which is termed dysbiosis [113], could spoil gut microbiota (GM) physiological properties leading to harmful effects to the human host [114].

Among the GM properties, there are important metabolic benefits such as improving the digestive functions. Bacteria allow the complete digestion of some food nutrients such as

fibers which otherwise cannot be metabolized by eukaryotic cells [115], and participate in the synthesis of some micronutrients [116]. Importantly, some relevant functions have been described for metabolic end products of microbial fermentation. For example, during metabolism of fibers, short-chain fatty acids (SCFA) such as butyrate, propionate, and acetate are produced [117]. The former is of great importance and acts as an energy source for colonic epithelial cells, thus contributing to the proper barrier function [118,119]. Besides its nutritional impact, the current evidence supports the fundamental role of the GM in the host defense. The intestine works as a boundary that separates the inner and the outer environment and the coexistence of microbial and somatic cells is highly mediated by the epithelial cells (EC). This complex system was well illustrated by Vaishnava et al. who emphasized the interplay between EC and gut microbes and its significance for their proper coexistence [120].

The mechanisms underlying the cross-talk between the gut microbial community and the IS are well stablished and it has now became clear the relevance of such interplay in the harmonious balance between the host and its microbiota [114]. The mucosal IS, which is distributed among the different levels of the mucosa layer, has to procure the right equilibrium between tolerance and reactivity, and T-cells are decisive for such balance [121]. Distinct T-cell sub-populations dominate in different gut locations, conditioning the immune activation through complex signaling pathways (28). Because of the impact of both commensal and pathogenic bacteria on the maturation of the IS, the study of the microbiota and gut integrity may clarify the field.

Today there is clear evidence of the relevance of an adequate development of the microbiota and immunity for the host wellness. Data from experimental studies on in vivo models have provided valuable knowledge. Findings from germ-free animal studies revealed important phenotypic and functional characteristics mediated by the intestinal microbes, and emphasized the importance of the microorganisms in the correct development of the human body structures [122]. Certainly, studies on the transfer of microbiota from humans or animal models to animals with known microbiota (gnotobiotic models) are prevalent and they demonstrate that some phenotypic characteristics are

dependent on the microbiota [123].

6.2.2. Microbiota and T1D

Vaarala et al. elegantly described the three main elements that may explain the connections between an altered intestinal track and T1D [124]. This triad includes a compromised gut permeability, immune dysregulation, and a dysbiotic microbial ecosystem. Additionally to the defective barrier function and intestinal environment, confirmed in later studies in T1D subjects [125], the microbes play a key role also in the development of T1D. For example, the number of anti-islet cell autoantibodies has been shown to correlate with some bacteria genera, suggesting that alterations in the microbiota composition may precede the pathology. Indeed some degree of gut dysbiosis has been observed in prediabetic subjects prior to T1D onset [126].

There is accumulating evidence of the role of GM in diabetic pathology. In fact, a divergent profile of intestinal bacteria has been reported in T1D individuals in comparison to non-diabetic subjects. A case-control study with a total sample of eight children, four cases and four controls, revealed that T1D patients possess distinctly different gut microbiota, compared to healthy subjects, characterized by an increased Bacteroidetes/Firmicutes ratio [127]. The same finding was reported in a later study on Chinese T1D subjects [128]. Giongo et al. emphasized that changes at phyla levels were essentially a result of shifts in specific genera; *Clostridiales* and *Bacteroides* in Firmicutes and Bacteroidetes, respectively. They also found a list of bacteria genera predominant in the diabetic and control children [127]. In a related publication, the same research group provided further findings regarding the GM composition in the same sample [129]. It should be noted the increased abundance of advantageous bacteria such as butyrate producing bacteria (BPBs) and mucin-degrading bacteria in healthy controls [129]. The former bacteria group is known to enhance the barrier function through the maintenance of the mucus layer as mentioned above. The later contributes with a better permeability by means of mucin production, aiding in a steady mucus layer as well as gut integrity [130].

A compromised presence of BPBs and the consequent decay of the barrier function is

thought to be a primary trigger of pro-diabetic intestinal profile. *Akkermansia* genus, specifically *A. muciniphila* is probably, along with the *Faecalibacterium* genus, the most studied BPB. This taxa is specifically associated with the mucus layer by participating in its regulation through mucin degradation and human studies showed an association of its depletion with compromised mucus integrity [131]. Besides its structural role, *A. muciniphila* may have an effect in the defense response and in vivo studies demonstrated a function in the immune regulation by the activation of immune cells [132]. Indeed, children with T1D presented an under-abundance of A. *muciniphila* compared to controls [130], in concordance with the compromised microbial butyrate production observed in the NOD mice [133]. The restoration of A. *muciniphila* representation in type 2 diabetic mice also triggered important phenotypic features along with improvements in the barrier function [132]. These findings suggested that A. *muciniphila* could be a key player in the prevention and management of aberrant microbiota associated with T1D and related autoimmune diseases.

Likewise, microbial diversity appears to be impaired in T1D. A study using samples from eight Finish children in which four case children later developed T1D and the other four were controls, revealed that the case children's samples had an unsatisfactory development in GM diversity, which did not become as complex as controls's and was more heterogeneous among cases [127]. The same finding was reported by Kostic et al. [134]. Giongo et al. emphasized the importance of a compromised phylogenetic diversity in the risk of developing autoimmune diabetes and set the basis of potential screening criteria. Additionally, some functional attributes of the microbiome has also been reviewed in relation to T1D. Brown's team went further and detailed functional differences between controls and cases [129], revealing a greater taxonomic complexity in the control group. Conversely, a reduced metabolic capacity found in cases was associated with lower microbial diversity and predominance of unwanted bacteria taxa such as those matched to a pro-inflammatory state [127,134].

Long cohort studies and randomized controlled trials such as FINDIA (Finnish Dietary Intervention Trial for the Prevention of Type 1 Diabetes), BABYDIET (in German infants),

TRIGR (Trial to Reduce IDDM in the Genetically at Risk) and TEDDY among others, offer valuable information regarding the natural history of T1D and the role of GM (reviewed in [123]). Within the findings, the effect of geographical location on intestinal microbiota has received considerable attention. Other in vivo studies have contributed with valuable knowledge as well. For instance, Kriegel and colleagues correlated the abundance of intestinal segmented filamentous bacteria (SFBs) with the development and progression of diabetes in NOD mice [135]. Although a protective role for SFBs could not be presumed, they concluded that SFBs somehow attenuates the progression of T1D and promotes a boost in some T helper cell sub-populations. SFBs were initially considered latent but the current evidence clues that they have a role in mucosal immunity and immune response.

The features and characteristics of a pathogenic T1D-prone microbiome seems to precede the disease, which offers a possibility to anticipate and prevent or delay T1D onset [118,123,134]. Therefore, the GM could be used as a potential marker for disease progression. For instance, some specific bacteria taxa, such as the Ruminococcaceae family, have proven to have an inverse relationship with the levels of serum hemoglobin A1c [128], a widely used biomarker for the evaluation of diabetes progression.

A large number of experimental and observational studies demonstrated the efficiency of both probiotic and prebiotics, as well as synbiotics and fermented products, in conferring benefits on the host [136]. Thought probiotic efficiency is specie-dependent, and some methodological and technical issues such as the dose or the capacity to colonize the gastrointestinal track may limit their efficiency [137], this approach seems promising for T1D. Along with the aforementioned dietary modulations, fecal transplants also offer a possibility of changing host's microbiota. The fecal microbiota transplantations (FMTs) were initially used in experimental studies [138] but has proven to be effective in the management of some intestinal pathologies [139] and its use in T1D has been discussed [140]. Despite the controversy about its use, FMTs may be a useful tool for immunomodulation and seems to be a promising approach for the GM modulation.

Some novel publications discuss the relevance of the aforementioned products for T1D management [141,142]. Interestingly, studies in mice models [143] and humans [144]

reported beneficial outcomes after intervention with potentially beneficial bacteria. For instance, the administration of the probiotic A. muciniphila showed an improved insulin sensitivity and glucose homeostasis, healthier lipid profile, and a pro-inflammatory tone among others changes. Interventions that aimed to promote A. muciniphila abundance through a prebiotic effect [144,145] offered positive effects as well.

6.3. JAK Pathway Inhibition

6.3.1. Introduction

The mammalian Janus kinase (JAK) family contains three JAKs (JAK1, 2, 3) and tyrosine kinase 2 (TYK2), which selectively bind different receptor chains [146]. Upon binding of ligand to its cognate receptor, associated JAKs become activated and undergo phosphorylation, which creates docking sites for the SH2 domain of the cytoplasmic transcription factors termed signal transducers and activators of transcription (STATs). The human STAT family contains seven STATs: STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6. Following phosphorylation, STATs are translocated to the nucleus, dimerize, and bind to specific DNA sequences to regulate gene transcription [147]. The JAK-STAT pathway plays a pivotal role for the downstream signaling of inflammatory cytokines, such as IFNs, ILs, and growth factors [148].

6.3.2. JAKs and T1D

A type I IFN signature precedes the detection of autoantibodies in children genetically at risk for T1D [149] and IFNα is expressed in human islets from type 1 diabetic patients [150,151]. MHC class I overexpression is induced by IFNα [152] and IFNγ [153] in human islets from T1D patients and IFNα also induces cell endoplasmic reticulum stress and chemokine production [154].

Receptor engagement by IFN triggers JAK1-TYK2 heterodimer signaling (**Figure 2**). TYK2 has been associated with several autoimmune diseases including rheumatoid arthritis and

T1D [155,156]. Six TYK2 single nucleotide polymorphisms (SNPs) (rs34536443, rs2304256, rs280523, rs280519, rs12720270, and rs12720356) have been explored in relation to autoimmunity. Crucially, the SNP rs2304256 causes a missense mutation in TYK2, and has been associated with protection against T1D [155].

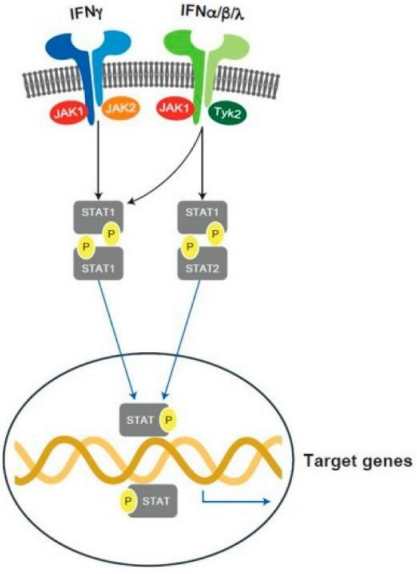

Figure 2. JAK1-TYK2 heterodimer signaling pathway

Downstream IFNα/IFNγ signaling is STAT1 dependent (**Figure 1**), and STAT1 is overexpressed in T1D islets and strongly correlates with HLA class I expression in β cells [153].

IFNγ is also involved in the expression of the CXCL10, which seems to be activated in islets from both T1D patients [157] and non-obese diabetic (NOD) mice [157,158]. CXCL10 promotes pathogenic T-cell infiltration into the pancreatic islets leading to β cell apoptosis

and its neutralization prevents diabetes in NOD mice [159]. A lack of IFNγ delays the progress of autoimmune diabetes in NOD mice [160].

Recent evidence further supports the rationale that IFN-driven JAK-STAT pathway activation significantly contributes to T1D pathogenesis. Patients with STAT3 gain-of-function germline mutations are susceptible to T1D with the median age of onset being 8 weeks. Furthermore, approximately 15% of patients treated with immune checkpoint inhibitors develop endocrine autoimmunity [161], including pancreatic β cell targeting [162], leading to T1D [163]. Consistent with these observations, inhibition of PD-1-PDL1 signaling accelerates diabetes in NOD mice [164].

Prior treatment of *in vitro* human islets with ruxolitinib (JAK1/2) significantly reduced IFN_ mediated inflammatory and ER stress markers [165]. Moreover, treatment of NOD mice with a JAK1/JAK2 inhibitor (AZD1480) blocked MHC class I upregulation on β cells and reversed autoimmune insulitis by reducing immune cell infiltration into islets in newly diagnosed animals [166].

Finally, pancreas-specific genetic knockout studies revealed an essential role for STAT3 in islet architecture, but it is dispensable for the function of mature islet [167,168]. In contrast, STAT5 is only important for age-dependent glucose intolerance [169]. These studies suggest that β cell function is minimally impacted by JAK-STAT pathway inhibition.

6.3.3. Summary

Taken together, IFN driven T1D pathogenesis can be potentially downregulated by inhibiting the downstream JAK-STAT pathway.

7. Concluding Remarks and Outlook

Diabetes is a complex disease that originates from dysfunction and destruction of beta cells as a result of a pathogenic response that involves both the adaptive and innate immune system [170,171].

During T1D development, T-cells seem to play a crucial role for destruction of beta cells [172]. Therefore, T-cells have been target of most immunotherapy strategies, dues to the main hypothesis that beta cells could survive by suppressing the pathogenic reactivity of specific T-cells. Although these strategies have demonstrated to be effective, unfortunately, the efficacy was short-lived. On the other hand, immunotherapy protocols based on specific antigens, such as vaccination with peptides derived from beta cells, should take into account the high degree of diversity in the response of specific T-cells against beta cells among individuals with T1D [173]. For this reason, the most effective approach should contemplate the combination of different strategies in order to allow the elimination of islet-infiltrating T effector cells through different mechanism. In this sense, new strategies with the objective of improving glycemic control are constantly investigated with the goal to address the long-term insulin dependence that leads to a poor quality of life.

In addition to immune interventions, other ongoing studies are investigating ways to restore insulin secretion using different approaches. It is important to note that, due to the heterogeneity of T1D, the future of T1D treatment strategies most probably would be in direction of a more personalized approach.

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~ *Study* 3 ~

Influence of Storage Temperature and Packaging on Bacteria and Yeast Viability in a Plant-Based Fermented Food.

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Abstract: Optimization of food storage has become a central issue for food science and biotechnology, especially in the field of functional foods. The aim of this work was to investigate the influence of diggerent storage strategies in a fermented food product (FFP) and further determine whether the regular storage (room temperature (RT) and standard packaging (SP)) could be refined. Eight experimental conditions (four different temperatures × two packaging) were simulated and changes in FFP's microbial ecology (total bacteria, lactic acid bacteria (LAB), and yeasts) and physicochemical characteristics (pH and moisture content (MC)) were determined following 1, 3, 6, and 12 months. All conditions tested showed a decline in microbial content due to the effect of the temperature, 37 ºC being the most detrimental condition, while -20 and 4 ºC seemed to be better than RT in some parameters. Vacuum packaging (VP) only had a major effect on MC and we found that VP preserved greater MC values than SP at 3, 6, and 12 months. The correlation analysis revealed that total bacteria, LAB, and yeasts were positively associated, and also both pH and MC showed a correlation. According to our results and with the purpose to maintain the load of viable microorganisms, we observed that the best storage conditions should contemplate SP and freezing or cooling temperature during a period no longer than 3 months.

Keywords: fermented foods; lactic acid bacteria; packaging; probiotic; storage; temperature; viability; yeasts

1. Introduction

The development of new functional foods has gained recent interest due to the growing incidence of chronic diseases [1,2] and the central role of nutrition in most of them [3,4]. Among functional foods, fermented foods are recognized as beneficial for humans' microbiota and are well established in the health market as promising therapeutic agents [5–7]. Fermented foods can be defined as foods and beverages produced through the culture of certain microorganisms in controlled conditions [8]. These fermentation processes involve substantial modifications in the food matrix that increase its nutritional value [9,10] and also provide unique organoleptic attributes [11] and useful technological properties [12]. When fermented foods are not subjected to further technological transformations, such as pasteurization or high pressure treatments [13,14], they can be used as vehicle for probiotics: "live microorganisms which when administered in adequate amounts confer a health benefit on the host" [15]. Although recent findings suggest that bacteria viability is not always necessary for producing beneficial clinical effects [16–18], major efforts have been directed towards maintaining the highest load of alive microorganisms at the time of consumption.

Although fermentation processes tend to increase food stability [19–21], during shelf life food resident microflora must cope with a list of circumstances which endanger their survival. Intrinsic and extrinsic factors that influence on the survival of probiotic species in foods include ingredients, physicochemical characteristics, processing, handling, and storage [22–29]. For instance, acidity is one of the most relevant factors. Most microorganisms grow well at pH around neutral (pH of 7) but an extremely acidic environment is a growth-limiting factor [30–32] and is responsible to a large degree for the loss of viability of probiotics [33]. Similarly, nutritional characteristics like water content [34,35], solutes [36], nitrogen [37], or fermentable sugars [29] are relevant aspects to be considered for the microbial metabolism.

Additionally, storage time and temperature can affect the bacterial survival [30,38–40] thus the manipulation of environmental temperature could be useful for reducing the loss of viable bacteria. In general, high temperature importantly decreases microorganism's

viability [41,42] while low temperature, like refrigeration, has been reported to be better for the survival of certain probiotics [43]. Other strategies for increasing the survival of microorganisms in foods, focus on minimizing oxygen exposure by manipulating the packaging, incorporating antioxidant compounds, or regulating the environmental light [24,30,44].

Most of the available information relative to probiotic survival comes from studies carried out on dried probiotics [22,42] or dairy products [45–47]. Nonetheless, how probiotic bacteria behave in other food matrixes have not been researched in depth. Moreover, because of the increasing demand of lactose-free and vegetarian foods [48], new-era food products have been developed during the last years and alternative food carriers from plant origin are being explored as vehicle for microorganism delivery [41,49].

The present research was carried out in a plant-based food, fermented by a combination of lactic acid bacteria (LAB) and yeasts. This food product, henceforth called FFP (fermented food product), is commercialized for animal production as a food supplement with functional properties (https://cordis.europa.eu/project/rcn/206082/factsheet/es; HEALTHSTOCK Ref. 733627). Findings from previous studies support that FFP is useful in enhancing performance and immunity in dairy animals [50,51], and a recently published study in a type 2 diabetic rat model revealed its potential anti-diabetic properties [52].

In the present manuscript we aimed to determine how storage impacts on the microbial load in FFP and whether different storage conditions alternative to the ongoing one (room temperature and standard packaging) would contribute with a better preservation of the alive microorganisms present in FFP. For this purpose, we conducted a comparative study to determine the influence of different storage conditions (four different temperatures and two packaging conditions) on FFP. Consequently, the findings would allow us to understand the influence of temperature and packaging during FFP storage.

2. Materials and Methods

2.1. Raw Materials and Production

The research was carried out on a fermented food product (FFP) including soya flour, alfalfa and malta sprouts, along with other minor components obtained directly from the manufacturers (Pentabiol S.L, Navarre, Spain; www.pentabiol.es/?lang=en). The appearance of the FFP is similar to fine sawdust and presents a mean particle size of 0.1 mm (**Figure S1**). During the production of FFP the first stage covers the fermentation of a mixture of pre-cultured starter microorganisms, including LAB and yeasts, with other minor components. The second phase includes the incorporation of this culture to the raw materials for a second fermentation. At the end, air drying is used to reduce moisture content in the final product.

2.2. Experimental Design

Experiments were run from the product fabrication (0 month) to its best-before date (12 months) including some intermediate time points (1, 3, and 6 months). The product was packaged in two different conditions and stored at four different temperatures. The effect of oxygen exposition was tested with the utilization of two different packaging conditions, standard packaging (SP) and vacuum packaging (VP) (**Figure S2**). The selected storage temperatures ranged from low temperatures (freezing at -20 ºC (F) and cooling at 4 ºC (C)) to high temperature (37 ºC (HT)). Additionally, room temperature (RT) was set with a portable measuring instrument (Humidity/Temperature Data Logger PCE-HT 71N, PCE, Spain). **Table 1** summarizes the experimental conditions employed and the samples coding. RT and SP were used as temperature and packaging reference conditions, respectively.

Table 1. Experimental Conditions and Sample Coding.

Experimental conditions		Sample code
Storage temperature	**Packaging mode**	
Freezing (-20 ºC)	Standard	F-SP
	Vacuum	F-VP
Cooling (4 ºC)	Standard	C-SP
	Vacuum	C-VP
Room temperature (22 ºC)*	Standard	RT-SP
	Vacuum	RT-VP
High temperature (37 ºC)	Standard	HT-SP
	Vacuum	HT-VP

*Data from the Humidity/Temperature Data Logger revealed that the temperature in the laboratory was 21.81 ± 2.2 ºC, so RT was set at 22 ºC. F-SP: freezing standard packaging; F-VP: freezing vacuum packaging; C-SP: cooling standard packaging; C- VP: cooling vacuum packaging; RT-SP: room temperature standard packaging; RT-VP: room temperature vacuum packaging; HT-SP: high temperature standard packaging; HT-VP: high temperature vacuum packaging.

2.3. Sample Preparation

Freshly produced FFP was portioned and bagged in individual packages containing 150 g of the product. Each experimental condition was replicated twice (and performed repeated measures) and individual bags were created for the measurement of each microbiological and physicochemical parameter to facilitate experiment execution. In order to mimic as close as possible regular sacks commercialized by the manufacturer, the same package (a three layer bag containing two paper layers and a plastic layer in between) and sealing technique (industrial sack sewing machine) was employed. Vacuum packaging was performed using polyethylene plastic bags and a vacuum sealer (Silver Crest, Hamburg, Germany). The final number of required bags was 256 (eight conditions x two duplicates x three parameters x four time points). With the purpose to ensure that we had the necessary samples, some extra packs were prepared and exposed to all the experimental conditions. See the experimental design scheme in **Figure S3**.

Before any test, all samples were adjusted to RT. Prior to every experiment, the content of the package was mixed thoroughly using a sterile spatula and the sample was analyzed

according to the different protocols. During sample handling gloves were used and working areas were sterilized with 70% alcohol. Contamination was avoided using gas burners.

2.4. Microbiological Analysis

Viable bacteria were determined by classical culture-based methods at each sampling time (0, 1, 3, 6, and 12 months). The amount of total aerobic bacteria (total bacteria), LAB, and yeasts was determined by using Plate Count Agar (PCA) (Sigma), de Man, Rogosa, and Sharpe agar (MRS) (Sigma), and Sabouraud Glucose agar with chloramphenicol (Sigma) mediums, respectively. All media were prepared following manufacturer's instructions, autoclaved at 120 ºC for 15 min and cooled to 42–45 ºC before use. For every sample a 1:10 dilution (extract) was prepared with 10 g of FFP and 90 mL of 0.85% sterile saline solution containing 0.1% of peptone from casein (Scharlau, Sentmenat, Spain). The mixture was poured in a sterile stomacher bag and homogenized for 2 min with a Stomacher (LB400 Homogenizer, VRW International). The resultant product was then transferred to a sterile glass bottle through the stomacher bag filter and serial 10-fold dilutions in sterile saline solution were prepared. All plates were inoculated by standard pour plate method (1 mL of sample solution and 20 mL of medium) except for MRS agar, which was cultured by spread plate method (100 mL of sample solution in 20 mL of solid medium), as recommended by the European Standard EN 15787:2009 for the isolation and enumeration of *Lactobacillus* spp. in animal feeding stuffs. All dilutions were plated in duplicate and two negative control plates were prepared for each medium. MRS plates were grown in the culture conditions referenced above (anaerobic incubation at 37 ºC for 72 h). PCA and Sabouraud plates were incubated as indicated by the European Standard EN ISO 4833-1:2013 (aerobic incubation at 30 ± 1 ºC for 72 ± 3 h) and ISO 7954:1987 (aerobic incubation at 22–25 ºC for 3–5 days), respectively. After the incubation period plates were counted and the average number of colony forming units (CFU) per gram of FFP was calculated. Data is presented as mean of duplicate determinations (plating) from a single extract. Plates containing less than 4 CFU were counted as <10 CFU/g of sample.

2.5. Physicochemical Analysis

The pH was measured at RT by electrode immersion with a pH meter Crison Model 2001 (Crison Instrument S.A., Barcelona, Spain). A solution with 10 g of the FFP and 90 mL of sterile deionized water was prepared in duplicate for each replica. Measurements were performed in triplicate in agitation with a magnetic stirrer to avoid sample sedimentation.

For the determination of the moisture content (MC) and according to the referenced international method available for cereals and cereals products (ISO 712:2009), 5 ± 1 g of sample was used and left to dry at 130 ºC for 2 h. Measurements were performed in duplicate for each replica. The percentage of water present in the sample was calculated using the given formula MC% = (m_0 - m_1/m_0) x 100, where m_0 refers to the initial mass and m_1 refers to the mass after drying.

2.6. Statistical Analysis

All statistical procedures were performed using SPSS software for Microsoft (IBMSPSS Statistics 20). Data from each sampling time (1, 3, 6, and 12 months) and parameter (total bacteria, LAB, yeasts, pH, and MC) were submitted to univariate analysis of variance (ANOVA) by using the generalized linear model (GLM). Comparisons were performed between the different categories of temperature and packaging and the reference conditions: RT and SP, respectively. The significance level was set to $p < 0.05$, and $p < 0.01$ and $p < 0.001$ were considered highly significant and extremely significant, respectively. Data are presented as mean ± standard deviation (SD).

The Spearman correlation analysis was performed and Spearman correlation coeffcient (ρ) was estimated to determine the linear association between the following variables pH, MC, total bacteria, LAB, and yeasts (n = 80). The outcome results were interpreted according to the degree of association as very high (ρ = 0.9–1), high (ρ = 0.7- 0.9), moderate (ρ = 0.5- 0.7), or low (ρ = 0.2–0.5) after taking significant correlation ($p < 0.05$) values into consideration.

3. Results

3.1. Dynamics of Total Bacteria and LAB Stored under Different Temperature and Packaging Conditions

The results for the effects of storage temperature and packaging mode on the counting of total bacteria in FFP are shown in **Figure S4**. Overall, FFP experimented a reduction in the load of total bacteria after 12 months of storage, that fluctuated between 8% and 44% in C and HT, respectively. F and RT had intermediate values (9% and 26%, respectively). Undoubtedly, F and C temperature were the conditions that preserved better the content of total bacteria in FFP, which experienced a reduction of only 0.47 and 0.40 log units, respectively, after one year of storage. On the contrary, HT presents the more challenging temperature condition for total bacteria because up to 2.09 log units were lost during the same period. When the effect of storage temperature was compared between the temperature conditions some significant differences were also found (**Figure 1A**). During the first 3 months the number of total bacteria in C and F temperature was comparable to that in RT ($p > 0.05$ at 1 and 3 months). At 6 months, however, C and F temperature had greater number of total bacteria than RT ($p < 0.001$ and $p < 0.001$ in F and C, respectively). At 12 months significance was only observed in C temperature ($p < 0.05$). The number of total bacteria in HT was smaller than RT in all the sampling points ($p < 0.01$, $p < 0.001$, $p < 0.001$, and $p < 0.01$ at 1, 3, 6, and 12 months, respectively).

In regard to packaging, total bacteria count in FFP was similar in SP and VP at all the sampling times, and statistical significance ($p < 0.05$) was found only at 6 months, the total bacteria load being lower in VP (**Figure 1B**).

Concerning viable LAB in FFP, some differences were found among the studied experimental conditions too (**Figure S5**). Baseline LAB load experienced a sharp decline after 12 months, with the exception of F temperature. At 12 months, samples at RT lost half of viable LAB content (53% of loss), samples stored at lower temperature (F and C) showed a slighter decline (12% and 39% of loss, respectively) while samples stored at HT suffered the greatest viability decrease (86%). Samples stored at F temperature only lost 0.93 log

units. Such decrease is small in comparison with the drops of 2.98, 4, and 6.44 log units found in C, RT, and HT, respectively. Indeed, samples at HT got the lowest LAB load at 12 months with < 1 log CFU/g, while the other conditions managed to keep values over 3.44 log CFU/g at that time.

Comparison of the survival of LAB between RT and the other temperature conditions demonstrated statistically significant differences at all time points analyzed (**Figure 1C**). In F and C temperatures the number of LAB was statistically significantly higher ($p < 0.001$) than in RT at 1, 3, 6, and 12 months. Indeed, at 12 months the counts of LAB in F temperature were high and considerably greater than the load found in the remaining temperature conditions, including C temperature. In the case of LAB in FFP, F condition is the most favorable one. On the other hand, HT had lower LAB counts than RT ($p < 0.001$) at 1, 3, and 12 months.

The packaging mode only had a subtle effect on LAB and statistically significant differences between SP and VP were only identified at 3 months ($p < 0.001$), the time in which SP presented 0.13 log CFU/g more than VP (**Figure 1D**).

3.2. Dynamics of Yeasts Stored under Different Temperature and Packaging Conditions

The obtained average values of yeasts are given in **Table S6**. Following 1 month of storage, the load of yeasts drastically declined in all the temperature conditions (2.16 log units in RT and HT, 1.94 log units in C) excluding F temperature (0.46 log units). Similarly, at 12 months C, RT, and HT had lost 2.16 log units and F had only lost 0.67 log units. These results account for 67% and 20% of loss, respectively.

Yeasts displayed some slightly different dynamics when FFP was exposed to different storage temperature (**Figure 1E**). RT and HT had a comparable effect on yeast survival and no statistically significant differences were found at any time. On the other hand, relevant differences between storage at RT and low temperature conditions were identified. F

temperature led to higher ($p < 0.001$) counts of yeasts at all the sampling times. For C temperature, no statistically significant differences were found at 1 month ($p = 0.05$), however, significantly lower values were found at 3 ($p < 0.05$) and 6 months ($p < 0.001$). At the end of the study only the F temperature differed from RT in yeast content. Focusing on the packaging mode, VP did not provoke differences in viability of yeasts in FFP (**Figure 1F**).

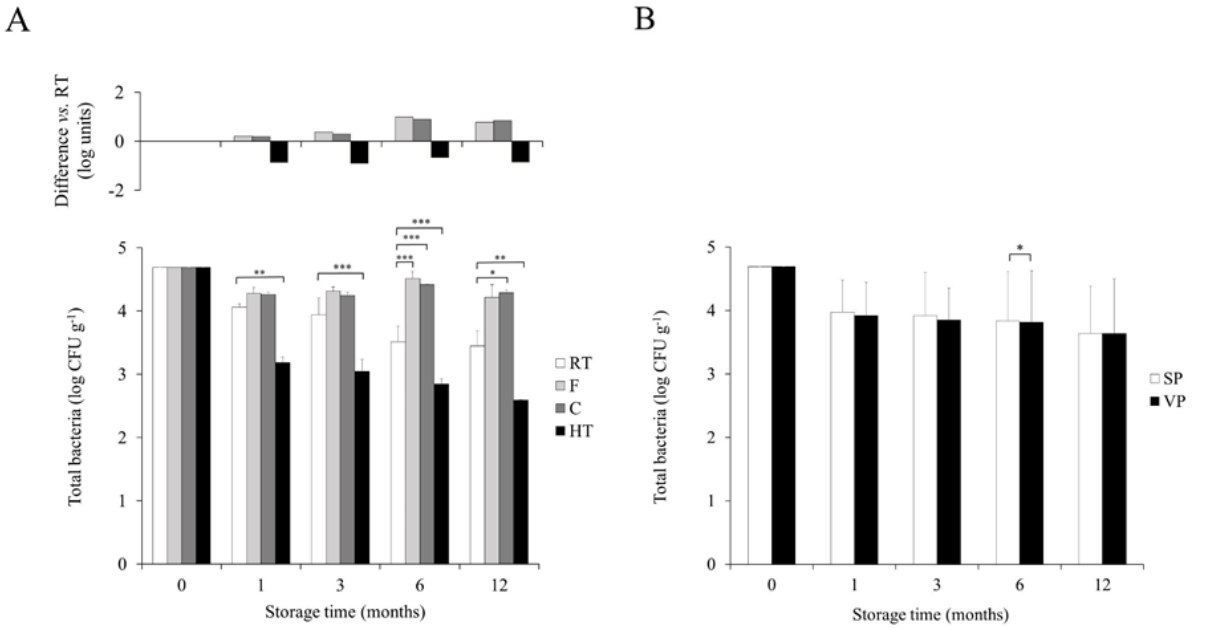

Figure 1A and 1B. Effect of Storage Temperature and Packaging Mode on the Counts of Total Bacteria (log CFU g^{-1}) in FFP Samples. FFP: fermented food product; RT: room temperature; F: freezing; C: cooling; HT: high temperature; SP: standard packaging; VP: vacuum packaging. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

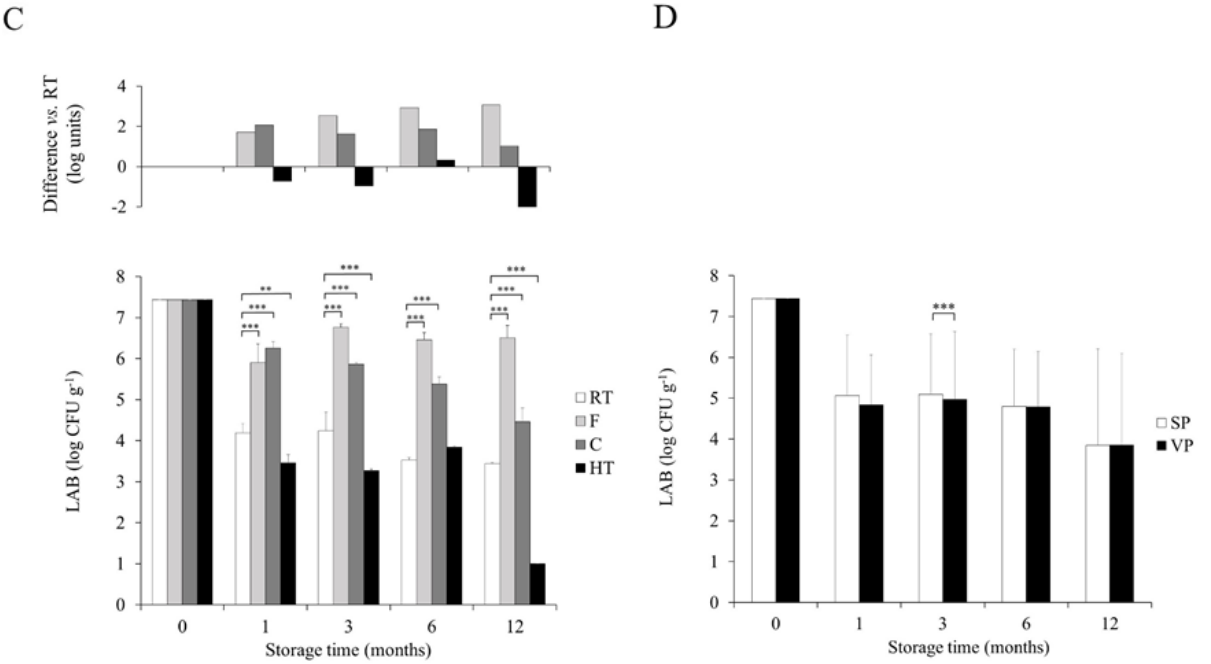

Figure 1C and 1D. Effect of Storage Temperature and Packaging Mode on the Counts of LAB (log CFU g^{-1}) in FFP Samples. FFP: fermented food product; RT: room temperature; F: freezing; C: cooling; HT: high temperature; SP: standard packaging; VP: vacuum packaging. * p<0.05, ** p<0.01, *** p<0.001.

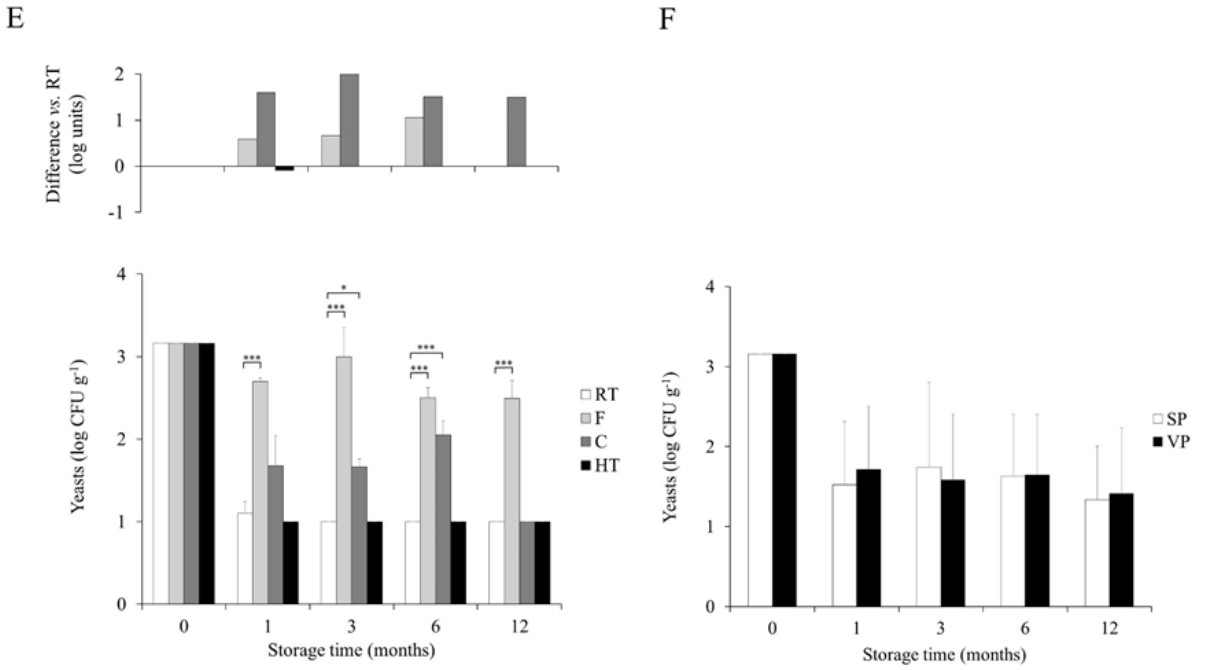

Figure 1E and 1F. Effect of Storage Temperature and Packaging Mode on the Counts of Yeasts (log CFU g^{-1}) in FFP Samples. FFP: fermented food product; RT: room temperature; F: freezing; C: cooling; HT: high temperature; SP: standard packaging; VP: vacuum packaging. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

3.3. The Influence of Temperature Conditions and Packaging Modes on pH

Values of pH measurements are summarized in **Table S7**. During the study and at the end of the study (12 months), the pH in all temperature and packaging conditions remained almost invariable in comparison to the initial pH value.

Concerning the storage temperature, only some differences were observed between FFP stored at RT and at low temperature (**Figure 2A**). pH in F and RT was comparable in all the sampling times except 3 months, where a decrease was observed ($p < 0.001$) in the former condition. In the case of C temperature significant differences with RT were observed at 1 and 3 months, being lower ($p < 0.05$) at 1 month and greater ($p < 0.001$) at 3 months in RT vs. C temperature. Statistically significant differences were not found between HT and RT at any time.

Packaging only showed to have a significant effect on FFP's pH values at 1 month, when VP presented a lower ($p < 0.05$) pH compared to SP (**Figure 2B**).

3.4. The Influence of Temperature Conditions and Packaging Modes on Moisture Content

The values obtained after MC determination are shown in **Table S8**. The degree of MC loss in FFP varied broadly from 5% to 70% of loss at 12 months and such loss was a gradual. Remarkably, a clear effect of temperature and packaging can be concluded since MC was very different between the eight samples.

When MC was compared between RT and the experimental conditions some differences were found at 3, 6, and 12 months (**Figure 2C**). F temperature was the condition which best preserved MC, and had greater values than RT from 3 months to the end of the study ($p < 0.001$ at 3 and 12 months; $p < 0.05$ at 6 months). With reference to C temperature, it showed higher MC than RT at 3 ($p < 0.01$) and 12 ($p < 0.001$) months but at 6 months the numbers were over RT values ($p < 0.01$). HT presented lower ($p < 0.001$) MC than RT at 3, 6, and 12 months.

With respect to packaging, during the study MC behaved almost identically in both packaging modes (**Figure 2D**). A gradual decline in MC occurred during FFP storage. No differences were found at 1 month, however, a considerable fall was registered between 1 and 3 months, after which MC remained almost unchanged (6 months) until a tiny final decline at the end of the study. Significant differences ($p < 0.001$) were found at 3, 6, and 12 months. At all the sampling time points VP preserved MC better than SP.

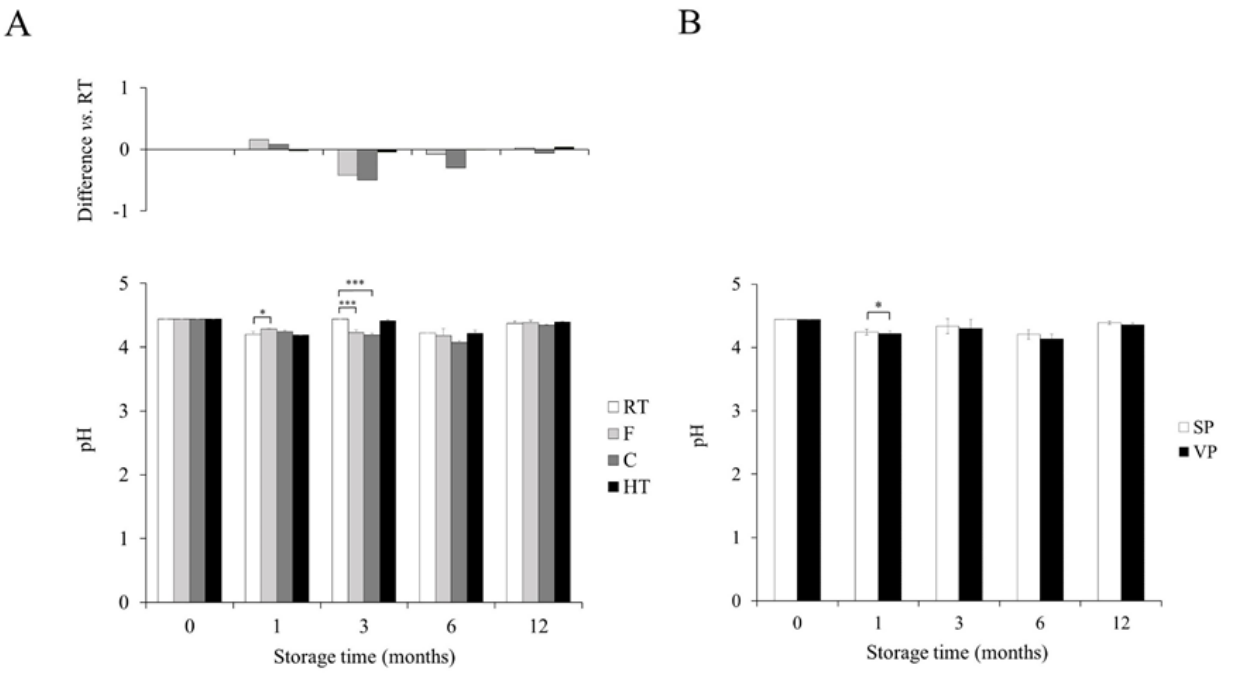

Figure 2A and 2B. Effect of Storage Temperature and Packaging Mode on pH in FFP Samples. FFP: fermented food product; MC: moisture content; RT: room temperature; F: freezing; C: cooling; HT: high temperature; SP: standard packaging; VP: vacuum packaging. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

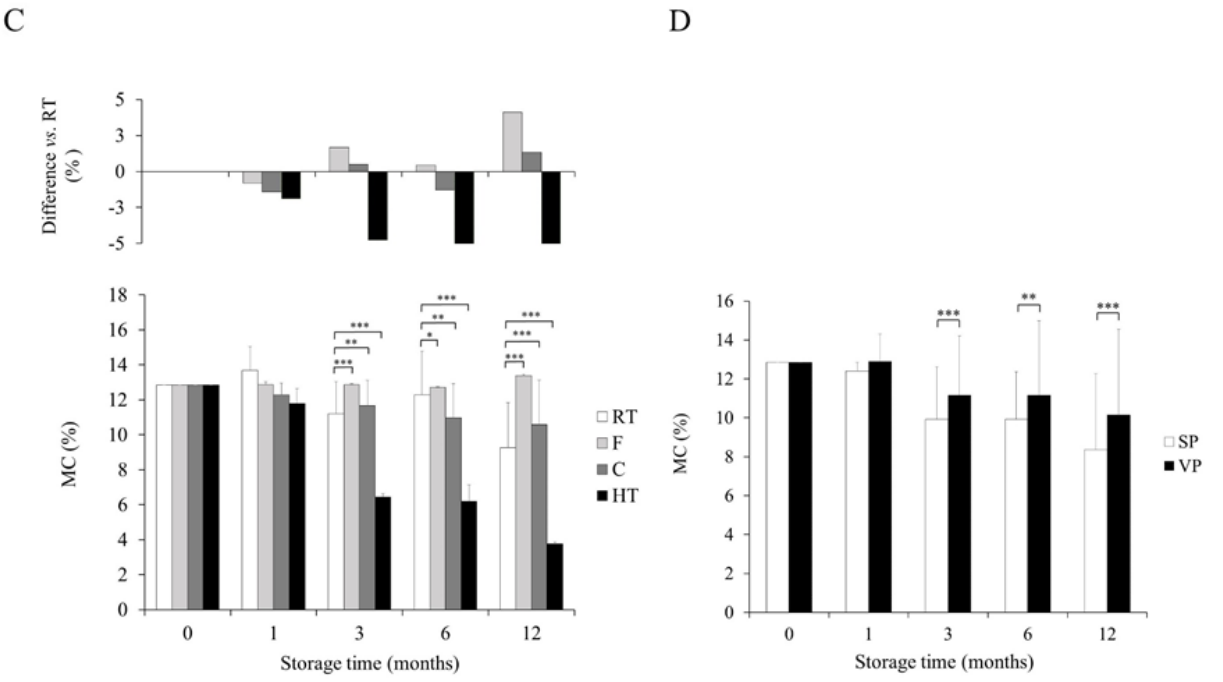

Figure 2C and 2D. Effect of Storage Temperature and Packaging Mode on pH moisture content (%) in FFP Samples. FFP: fermented food product; MC: moisture content; RT: room temperature; F: freezing; C: cooling; HT: high temperature; SP: standard packaging; VP: vacuum packaging. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

3.5. Interplay between Physicochemical and Microbiological Profile

Descriptive statistics of Spearman´s correlation coefficient (ρ) and the p-value are depicted in **Table 2**. Spearman´s correlation analysis revealed the statistically significant low positive correlation between pH and total bacteria (ρ = 0.228; p = 0.042), pH and LAB (ρ = 0.262; p = 0.019), and pH and yeasts (ρ = 0.293; p = 0.008). Similarly, a moderate positive correlation was observed between MC and total bacteria (ρ = 0.557; $p < 0.001$), MC and LAB (ρ = 0.618; $p < 0.001$), and MC and yeasts (ρ = 0.616; $p < 0.001$). Moreover, the analyzed microbiological profiles showed a high or very high positive correlation between them, total bacteria and LAB (ρ = 0.876; $p < 0.001$), total bacteria and yeasts (ρ = 0.846; $p < 0.001$), and LAB and yeasts (ρ = 0.913; $p < 0.001$).

In regard to pH and MC, a statistically significant correlation was not found between the analyzed physicochemical parameters ($p = 0.648$).

Reports of model coefficient values of total bacteria, LAB, yeasts, pH, and MC are available in **Tables S1–S5**.

Table 2. Spearman´s Correlation Coefficient (ρ) and its Level of Significance (*p*-value) for the Analysed Physicochemical and Microbiological Parameters.

	pH		**MC**		**Total bacteria**		**LAB**		**Yeasts**	
	ρ	*p* value	ρ	*p* value	ρ	*p* value	ρ	*p* value	ρ	*p* value
pH			0.052	0.648	0.228	0.042	0.262	0.019	0.293	0.008
MC	0.052	0.648			0.557	0.000	0.618	0.000	0.616	0.000
Total bacteria	0.228	0.042	0.557	0.000			0.876	0.000	0.846	0.000
LAB	0.262	0.019	0.618	0.000	0.876	0.000			0.913	0.000
Yeasts	0.293	0.008	0.616	0.000	0.846	0.000	0.913	0.000		

MC: moisture content; LAB: lactic acid bacteria.

4. Discussion

The main purpose of the present study was to draw attention to how storage conditions influence the microbial community present in FFP. The first variable that we considered analyzing was the load of viable microorganisms in FFP measured in specific microbiological media. Secondly, given that the nature of the food component can compromise microbial survival [23,25], the most important physicochemical parameters were also monitored and their influence on the microbial load was evaluated. Some authors had previously listed the key factors on probiotic viability [23,24,53] and with the exception of food processing, which was beyond the scope of this study, we have addressed most of them: characteristics of the food matrix, product packaging, storage condition, and microbiological profile.

In the present work we aimed to monitor the potentially beneficial bacteria load in FFP as previously determined in other food carriers [46,54–56]. Although the microorganisms in FFP resisted production and manufacturing and do not seem to be extremely sensitive to external agents [57], our findings revealed a reduction in the initial load. We presume that it was originated by changes in nutrient availability [29,58], exposure to products of the metabolism [56,59], and interactions within other microbial species [60,61], which can concurrently be motivated by external factors such as storage temperature, packaging, and time [44,62].

4.1. Bacterial Viability in FFP

As above mentioned, environmental temperature is a key regulator of microbial survival and can be deleterious for bacteria stability [22,56]. Hypothetically and in agreement with the available scientific evidence [24,63], the most suitable temperature for the survival of microorganisms in FFP would be low temperature: freezing or cooling. According to our results and focusing on total bacteria, for a short storage time (3 months or less), storage at low temperatures (F or C) does not have advantages over RT, being that both had comparable counts of total bacteria. For storage periods longer than 6 months, however, it

would be better to store FFP at F or C temperature. Regarding LAB, they were more sensitive to storage than total bacteria. Following 1 month of storage low temperatures were better than RT for LAB's survival. It appears that F is the most convenient condition, far better than C. Our findings share a number of similarities with earlier studies which reported that low temperature is helpful in preserving the microbial load [29,34,63,64].

4.2. Yeast Viability in FFP

In spite of the fact that bacteria have received the most attention as probiotic microorganisms, yeasts present an alternative or complementary source with probiotic effects [65] and contribute with a number of technological properties of substantial interest in food production [66]. In contrast to bacteria, there has been little discussion on the stability of yeasts in food products and reports on the cell counts of yeasts through storage are scarce. Clearly, storage at 37 ºC or above results in detrimental viability of prokaryotic and eukaryotic microorganisms in FFP. This could be attributed to the great impact that high temperature has on the water content, which may indirectly compromise microbial viability as hypothesized by other authors [67].

4.3. Interplay between Microbial Groups

In complex mixtures of microorganisms like some fermented foods, the presence of specific microbes can modify the final balance with a beneficial or deleterious effect [8,60,61]. Some microorganisms can promote the survival of others through the liberation of growth-promoting factors to the media [31,68]. For instance, some published reports indicate that the presence of yeasts is favorable for the maintenance of LAB viability, probably because of their nutritional properties [39,69,70]. On the other hand, the combination of both LAB and yeasts may be detrimental for the latter, since some LAB-derived molecules or metabolites such as acetic acid [57] or bacteriocins [71] showed an antifungal activity [30,65]. It has also been reported that in situations in which both yeasts and bacteria

coexist in the same matrix, conditions of high pH (above neutral pH) are especially damaging for the former, which suffer a decline in their growth because of the competitive advantage of bacteria [72]. Considering that, it is likely that some interactions happened between bacteria and yeasts that coexist in FFP. Our data pointed out that LAB, total bacteria, and yeasts showed a high positive correlation, so it could be speculated that there was not an inhibitory or competitive exclusion between bacteria and yeasts in FFP's ecosystem.

4.4. Minor Effect of Packaging Mode on FFP's Microorganisms

Besides environmental temperature, exposure to oxygen is another relevant parameter to take into consideration for bacterial survival and growth. Generally, oxygen has a detrimental effect on bacterial survival either directly with peroxidation reactions [24] and generation of products [59], or indirectly, by affecting adjacent cells [30]. Oxygen conditions inside the experimental packs was expected to vary between standard and vacuum packaging, and consequently influence differently on the viability of the resident commensal microbes. It is somewhat surprising, however, that our results did not reveal great differences between both packaging conditions. In all the analyzed microbiological groups (total bacteria, LAB, and yeasts), vacuum packaging did not provide an advantage over the conventional packaging mode. On one hand, it is plausible that vacuum packaging failed to maintain an anaerobic environment and residual oxygen remained in the product. This situation could be caused by the relatively high permeability of polyethylene, the material used for vacuum packaging, in comparison to other packaging materials [24,53].

On the other hand, it is also likely that the oxygen exposure between packaging conditions was different, however, it did not provoke adverse consequences on the bacteria survival, as previously reported in yogurt [73]. To confirm the role of oxygen and elucidate this issue, a study on the existing dissolved oxygen in SP and VP would be valuable

4.5. pH and Moisture Content in FFP through Storage

On the grounds that environmental conditions have a main effect on the growth kinetics of bacteria culture [29,34,39,63], we considered that the study of pH in FFP would be valuable for the understanding of what happens on the product during its storage. It is generally accepted that a decline in a pH value could be an indicator of favorable conditions for bacterial survival, as the activity of viable microorganisms can be responsible for changes in pH in the product [46], probably because of the production of organic acids [60,74]. Conversely, an extremely low pH is generally associated to a reduction in the growth yield [39] because it can lead to undissociated acids [26,30]. In FFP the load of microorganisms decreased over time, however, FFP´s pH hardly changed besides its positive correlation with total bacteria, LAB, and yeasts. It could be due to the buffering effect of the matrix, as previously reported in a beverage with milk and carrot juice inoculated with probiotics [64].

Studies on other food matrixes did observe an acidification through storage, which is hypothesized to be caused by residual microbial activity. Yogurt stored at 5 ºC suffered from reductions of 0.2–0.5 units in pH and the loss was dependent on the probiotic species studied [46]. A study on cheese inoculated with probiotics revealed that pHwas stable during 29 days of storage at 4 ºC, however, when the storage was at 12 ºC a significant acidification occurred in the samples. Again, the change was dependent on the inoculated probiotic bacteria [68]. The authors suspected that the indirect stimulation of bacteria viability by microbial metabolites may explain pH reduction. For example, in dry fermented sausages, pH significantly increased through 120 days storage under different temperatures (4, 22, and 37 ºC), and the storage at 37 ºC had the biggest impact on the pH [74]. Other products like boza [49] or some fermented dairy products [46] had a significant drop in pH even when stored at cooling temperature. These findings suggest that is more than likely that the nature of food ingredients governs how acidity changes through storage.

Likewise, we considered that MC could be somehow relevant for the viability of microorganisms so it was explored as another physicochemical parameter. The water

content in a food matrix has a clear direct effect on the pressure of the cell walls and determines the osmotic pressure, which may be detrimental for microbial viability [34,35] and is a strong growth-limiting factor for yeasts [75]. The water content is of special interest in frozen or freeze-dried cultures [23,76,77], however, less information is available regarding how water present in a food matrix influences microbial survival.

4.6. Overall Influence of Storage on FFP

Figure 3 summarizes the overall influence of storage temperature on total bacteria, LAB, yeasts, pH, and moisture content in FFP samples following 12 months of storage. As noted above, temperature had a considerably greater impact on FFP than packaging mode. The analysis performed suggests that high temperature had a greater effect on all the analyzed parameters, while lower temperature preserved baseline values better. The adverse effects of high temperature on the survival of the alive microorganisms seems to be proportional to the storage time. Even though food distribution normally takes a few months, preventive actions should be taken to ensure that transport, shipping, and manipulation of FFP do not expose the product to high temperature. Moreover, when possible, cold chain must be set in order to impact as little as possible the alive microorganisms present in FFP.

To conclude, we can propose the optimal storage conditions for FFP according to the results obtained. On the grounds that LAB present interesting beneficial effects on the host [6,78] it would be advised to prioritize the survival of LAB over other bacteria groups. Hence, the storage of FFP at F or C temperature as long as possible would be recommended.

Besides, in cases where storage at low temperature is not feasible, it would be advisable to store. FFP protected from the light exposure and to consume it in a period of time that does not exceed 3 months. Regarding packaging, vacuum packaging did not show a protective effect on bacteria and yeast survival. Therefore, for the storage of FFP standard packaging would be as useful as vacuum packaging.

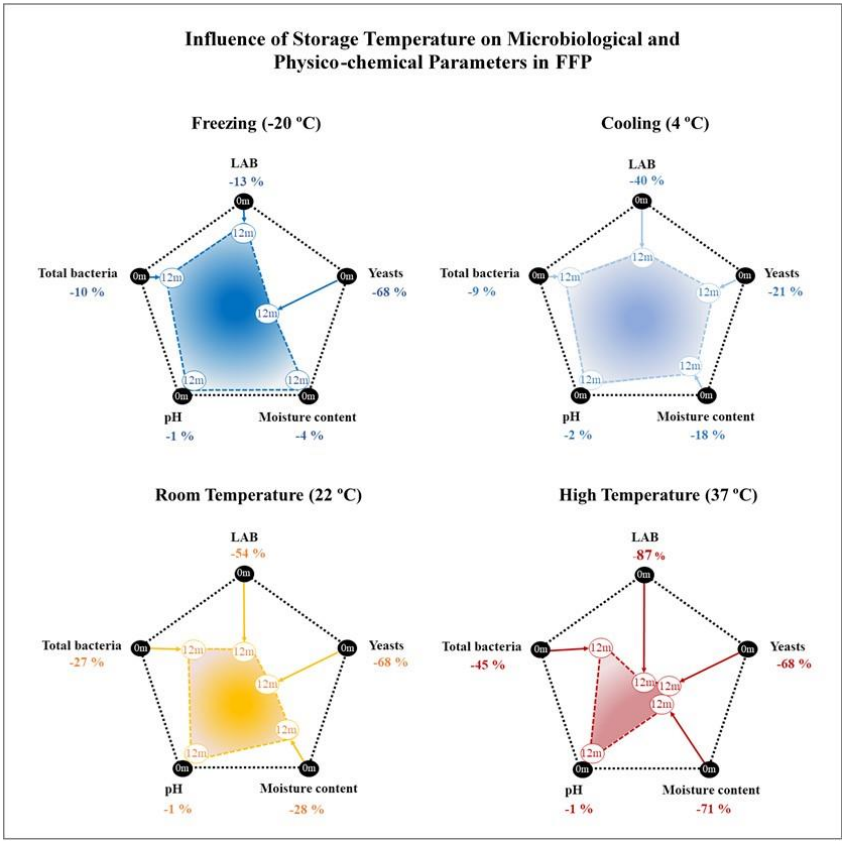

Figure 3. Overall Influence of Each Temperature Condition on the Microbiological Profile and Physicochemical Properties of FFP Following 12 Months of Storage. 0m refers to values at the beginning of the study; 12m refers to values at the end of the study for each temperature condition. FFP: fermented food product; LAB: lactic acid bacteria.

~ Graphical abstract and Supplementary materials are available in **Annex, pages 271-283.**

5. Conclusions

In summary, our findings showed that some procedures may be helpful in protecting the viability of FFP's microbiota, though the load of bacteria and yeast decreased through storage. Specifically, in relation to the storage temperature, storage at −20 and 4 ºC were the most convenient conditions and therefore would be recommended. Besides, taking the results into consideration, not exceeding a period of 3 months to preserve a substantial number of viable microorganisms would be recommended. Regarding the packaging methods, vacuum packaging revealed to not be better than standard packaging.

This work has led us to conclude that FFP is a relatively stable fermented food product for livestock which could be a suitable matrix for probiotics. Therefore, FFP and other plant-based fermented products with similar characteristics may be useful as novel probiotic delivery systems.

It should be noted that the present research was only an attempt to understand the dynamics of the complex microbial ecosystem in the FFP matrix. Given the clinical and technological relevance of bacteria identification up to strain level and the characterization of bioactive metabolites in foods, future studies with genomic and metabolomic approaches should be conducted to deepen understanding of the dynamics that take place in the FFP matrix.

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~ General Discussion ~

In the following section, the implications of the obtained results (*Studies 1*, *2* & *3*) are combined together, and the possible applications of these results are discussed.

Applications of Probisan® in Diabetes Mellitus

The growing incidence of T2D and other chronic diseases experienced has become a major concern for public health worldwide since they involve major economic costs and burden for patients (360,404,405). Even though considerable effort has been invested into valid treatments and efficient preventive strategies, current approaches do not seem to be entirely efficacious. Fortunately, recent GM investigations have unveiled the putative role of the intestinal microorganisms in our health, and GM-driven therapies look very promising for the management of DM (317) and other diseases (406).

In line with this rationale, the current thesis has focused on the role of a particular FF in T2D. This type of food offers new possibilities for the enhancement of overall health, the reduction of the risk for developing many diseases and, furthermore, it has been already proven to be useful for the clinical management of DM (97,407,408). Interestingly, one of the mechanism behind the functional claims of those products is the modulation of the GM (34,303).

As described throughout the whole manuscript, the GM has a central role in the current thesis. It possess a huge number of functions and exerts considerable influence on the host's fitness (218,254,279). Considering its dynamic behaviour (409) and the strong influence of dietary factors (292–294), in the *Study 1* we hypothesized that the Probisan®-supplemented group would show deep GM structural changes in comparison to control group. Contrary to what it was expected, we failed to observe meaningful differences in GM between groups, despite it was a long-term (31 weeks) dietary intervention that induced differences in other parameters like glucose homeostasis, intestinal glucose uptake or life

expectancy. In addition, we were unsuccessful to find consistence with previously published works, as discussed in the publication (98).

At first sight, our findings in *Study 1* may reveal a structural resilience of the GM in supplemented ZDF rats. Nevertheless, we should take into consideration some methodological issues. Firstly, we characterized the faecal microbiota by analysis of stool samples, that is a non-invasive method widely used for this purpose. Nonetheless, it is a surrogate of the intestinal microbial community and has some limitations (i.e., may include human DNA and other extraction-method bias (410), ignores the spatial organization of the GM (biofilms) (411), many microorganisms are anaerobes and could die or be damaged during sampling or storage (411)). Alternative invasive procedures such as the study of the cecum content (412) or biopsies from different regions of the intestine or its mucosa (410) would provide a more comprehensive characterization of the host's GM (413). Secondly, in our study we assessed the composition of GM by comparing the microbiome profiles obtained with 16S rRNA sequence-based metagenomics data but, unfortunately, this approach presents some limitations. In turns, it is highly recommended the use of whole-metagenome (metatranscriptome) sequencing (410). In addition, we placed more emphasis on the bacterial community, which is the most studied one, whereas we ignored other GM microorganisms such as fungi, viruses, protists and archaea (186). The intestinal fungal community illustrates this point clearly. Although it has received less attention thus far and the information available is extremely scarce, the study of the fungal community (mycome) through the sequencing of the ITS region looks promising (293), and may provide with very valuable knowledge on the role of the microbiome in T2D and health (376). Similarly, more research in the collection of intestinal viruses (virome) would enhance our knowledge on the topic (193).

In our research, we focused on the GM composition but we did not explore other microbiome signatures. Nowadays an extensive range of omics technologies are available that are particularly appropriate for the study of the GM, especially when combined. Overall, they allow a comprehensive investigation of the functional features of the intestinal microbial communities. To illustrate, transcriptomic patters obtained by

metatranscriptomic analysis reveal the actual gene expression and can reflect enrichments or declines in certain metabolic pathways (414). In order to gaining greater knowledge of the activity of the GM, we could also study the profile of proteins or proteome of the whole community using metaproteomics, or the microbial metabolites through metametabolomic analysis (415). The aforementioned techniques will reveal if the supplementation with Probisan® induces or not alterations in the microbial performance.

Yet, despite all setbacks, it is possible that our intervention did not provoke any change in the GM, or, it did but the alterations did not persist during the whole study and came back to its initial status. It has been hypothesized that the GM has memory and despite its exposure to profound environmental changes, for instance dietary changes, it is likely it would try to return to its original state (197,292). There are theoretical grounds for believing that a "fastidious" core microbiome is behind this resilience (416,417). If true, this phenomenon may explain the unexpected outcome reported in the *Study 1*. One possible implication is that the GM in the treated animals was resilient and moved toward its initial shape at some point during the study. This issue may be elucidated with the incorporation of several sampling times throughout the study and the reduction of the duration of the intervals within sampling points. It would reveal the dynamics of the microbial communities over time and identify transient effects otherwise ignored.

Regardless of the surprising results of the GM, in the *Study 1* we found very interesting outcomes. We could demonstrate that the administration of Probisan® *ad libitum* is favourable for the glucose metabolism and positively impacts on the host's health by protecting from diabetes-related complications and extending overall life expectancy. Conversely, other studies carried out in ZDF rats (418) and prediabetic subjects (419) found changes in GM structure following a dietary intervention with prebiotics that did not relate to phenotypic changes. That is to say, they reported GM changes that did not provide health improvements. In this regard, researchers should not focus exclusively on variations on the GM.

In this study, we decided to use a preclinical model for T2D. Nevertheless, according to our concluding remarks in the *Study 2*, and cumulative evidence of the influence of GM in many

pathologies, the same study could be repeated in experimental models of other diseases. In this line, further studies may be performed to investigate not only other forms of DM like T1D (400) (we have already completed an experimental study with Probisan® in NOD mice, oral communication, *unpublished work),* but also other chronic diseases, such as obesity (185), gastrointestinal conditions (253) or liver disease (420), or risk factors such as the metabolic syndrome (227). It is very likely that Probisan® would offer health advantages in some of these conditions.

Stability of Probisan® During Storage

The major aim in the *Study 3* was to describe Probisan®'s behaviour during its storage. Probisan® is used in a standardised manner in livestock production, has revealed health benefits in murine models and may have a potential use in the clinical setting. For that reason, it seems important to control how the product changes over time.

In the field of food science, and particularly in the study of functional foods, the characteristics of the food products are crucial because they are usually associated to their nutritional and functional properties. Besides, all food products should preserve a minimum quality during their shelf life. Most producers aim to develop products shelf stable at an ambient temperature (around 21-22 ºC), nevertheless, for some food groups it is very difficult to be stocked at that temperature without experiencing major changes in their attributes or functionality.

The *Study 3* was realized in the frame of the project HEALTHSTOCK (Ref. 733627) funded by the European Union Research and Innovation Programme Horizon 2020 (H2020), and was the first study evaluating the behaviour of Probisan® and its microbial ecosystem over time. It was not a shelf life study strictly speaking, since we did not study quality depletion and we did not address consumers acceptability (421). Instead, we did monitored changes in the microbiological profile as a function of time. This work tried to describe the changes occurring in the microbial groups to predict how they maintain or decrease their number through storage. Similarly, it was also the first attempt to explore how storage temperature

and packaging mode influence on both physicochemical and microbiological attributes, by mimicking the "standard" storage (room temperature and standard packaging) and comparing this with alternative conditions.

We gave especial importance to the load of viable microorganisms because we thought of them as the major responsible for the beneficial effects of Probisan® (although this issue remains to be tested and is discussed in greater detail below). On this basis, it is preferable to keep a larger bacteria and yeasts population in the product. Although the conditions that best preserved the microbial viability according to the *Study 3* required storage at low temperature, storage at room temperature also showed to preserve the load of viable microorganisms to some extent. This result is of economic significance given that refrigeration and freezing are associated with higher transport and storage costs for both industry and consumers. In the same way, the lack of significant effect for the vacuum packaging will simplify the handling and storage of the product, and will prevent from the use of more intricate packaging modes.

Besides, taken together, our findings suggest that there be may some protective elements in Probisan® that favour microbial viability. An illustration of this feature is given by the results of viable counts in those samples subjected to freezing. Despite those microorganisms were stored at very low temperature (-20 ºC), a substantial amount of bacteria remained viable even following 12 months of storage. Indeed, Probisan® presents a number of attributes that make it a good candidate for probiotic delivery. It includes fermented grains, which are better for probiotics than non-fermented grains (67), probably because of their source of nutrients (136). Further, though the particle size is small, Probisan® presents a solid matrix, that is more suitable for microbial viability than a liquid medium (159).

Additionally, malta, one of the main raw materials in Probisan®, have shown to be an excellent carrier for probiotics, probably because of the greater presence of compounds which can be used as nutrients for the microorganisms (422). Moreover, malta and the other raw materials used for Probisan® production, that is, soya and alfalfa, might also be a valuable source of compounds which could be protective for the survival of certain

bacteria strains. Indeed, soya (67), alfalfa (423) and malta (424) have been reported to be a source of antioxidant compounds such as phenolic compounds. Besides the considerable increase in the nutritional value of a given product (425), a high presence of phenolic compounds could be advantageous for the growth of some bacteria strains as previously investigated (426), for instance through the mitigation of the adverse effect of oxygen exposure (85).

Another major finding from the *Study 3* was the steadiness in pH values in the product in all the storage conditions. This is important, because as we reflected in the introduction and discussion, the acidity of the food matrix can compromise the microbial metabolism. Our results suggest that the inherent microbiota present in Probisan® does not compromise the characteristic of the substrate in spite of keeping its viability. To illustrate, even though we quantified yeasts and molds, the presence of molds was negligible, even when Probisan® was stored at room temperature or 37 ºC. Given our data, a plausible explanation for such absence of environmental mold contamination in Probisan® could be the presence of alive or inactivated bacteria and/or microbial derived products with protective effect againsts microbial contamination.

Inevitably, the *Study 3* has some constraints. Regarding the methodology, we analyzed the microbial content using classic culture-based methods. This technique has been extensively used for counting active microbes yet several weaknesses have been reported, including the underestimation (427) or overestimation (85) of the real microbial load. To illustrate, this technique may omit "viable but non-culturable" cells which are still viable (427). Another example could be that, since LAB can create greater structures and form chains, the use of a culture-based method may lead to misleading counts of CFU when some bacteria uncouple and get free from the chain (85).

It is important to emphasize that in the *Study 3* it was contemplated the use of a molecular-based method for a more specific quantification of microorganisms in Probisan®. Specifically, we decided to use a nucleic acid amplification technique by implementing Real-time Polymerase Chain Reaction (RT-PCR) (428–430). The reason to use RT-PCR was twofold. On one hand, due to the fact that the capacity of microorganisms to survive is

specie and strain specific (43,154), we presumed that not all the bacteria and yeasts strains would behave in the same way during Probisan®'s storage. On the other hand, investigations on health promoting microorganisms, mostly probiotic species, have unveiled strong evidence for strain-specific properties (110,431–433), highlighting the relevance of microbial identification up to strain level.

This assay is frequently used for the microbial profiling of food products among other practices (155,429,434). We developed a specific protocol for the DNA extraction in Probisan® and purchased all the reagents. Then we completed the DNA extraction, in duplicates, of all Probisan® samples at all the sampling points, and stored them until the analysis. Unfortunately, due to practical limitations we were not able to perform the RT- PCR reactions until the end of the study. At that time, the trial revealed the existence of technical problems, probably originated in a defective DNA extraction (i.e., presence of inhibitors, insufficient amount of DNA or damaged DNA template) and we were unable to perform the amplification of the DNA samples. Consequently, despite all the efforts, we could not obtain any results from this parameter.

Without any doubt, the use of RT-PCR would have provided with complementary information and more importantly, would have revealed the most appropriate storage condition for a given bacteria or yeast strain. Considering the health implications above mentioned, obtaining such information is priceless and would allow the optimization of Probisan® storage according to its intention of use.

Along with storage temperature, the packaging mode was the second main tested factor. We considered that the lower the oxygen exposure, the better for the microbial viability in Probisan®, so we attempted to reduce the amount of oxygen by the use of vacuum packaging. Contrary to previous works (118), we did not report an increased microbial viability. The lack of quantitative data on the real amount of oxygen in those samples stored under vacuum did not allow us to conclude whether oxygen is an important factor mediating in the survival of microorganisms in Probisan® during its storage. To confirm the role of oxygen and clarify this matter, it would be recommended to study the existing dissolved oxygen in the package and the gas permeability of the packages (148). Alternative

packaging materials, such as the multilayer Nupak™, an oxygen-barrier material, have previously been used to control the oxygen exposure in foods containing probiotics (175,176). Nevertheless, this issue should not be addressed until the role of oxygen in Probisan®'s microorganisms is elucidated.

Integrating Studies 1, 2 & 3: An Insight into the Possible Health-Promoting Components in Probisan®

On first thought, it would appear that the nature of the two studies on Probisan® (*Studies 1* and *3*) is disparate, however, they collectively contribute to the understanding of the food product. Both *Study* 1 and *Study* 3 were performed during a long period, and although they were not simultaneous, both used Probisan® that was expressly manufactured to that end. The Probisan® used for the *Study* 1 was stored in the animal-house and was maintained at an ambient temperature of 22 ºC during the whole study. That matches the condition of room temperature and standard packaging simulated in the *Study* 3. Nevertheless, Probisan® format in the *Study 3* was micronized (from now on referred as Micronized Probisan®), in contrast with the granulated formulation used in the *Study 1*, and such differences may influence the way the microbial ecosystem behave in the product. To get the most from the experimental desing in the *Study 3*, and infer the number of alive microorganisms in the Probisan® fed to the ZDF rats in the *Study 1* (from now on referred as Probisan®-1), we decided to include an additional sample of granulated Probisan® (from now on referred as Granulated Probisan®), that would allow the comparison between formats (micronized and granulated) and provide us with further information. To do this, we analysed, in duplicates, the microbiological profile of the Granulated Probisan® through storage under normal condition (room temperature and standard packaging) alongside the experiments in *Study 3* (we did not measure the remaining parameters, pH and moisture content, in order to avoid an overload of work).

Assuming that both produced batches (granulated product in *Study 1* and *3*) share properties and characteristics, we infered the number of alive microorganisms in

Probisan®-1 from that results observed in Granulated Probisan®. It is important to keep in mind that such inference presents some limitations: the two products were not produced at the same time, they were not stored in the same physical space, and the sampling points differ between studies. Nevertheless, this approximation can help us to take a step forward the mechanisms of action of Probisan®. **Figure 9, 10** and **11 (pages 198-200)** offer a schematic illustration of the load of viable microorganisms (measured as total bacteria, LAB and yeasts, respectively) found in the Granulated Probisan® plotted against the levels of FBG measured in ZDF in the *Study 1.*

The figures reveal some degree of loss of viability in all the microorganisms from 1 month of storage, especially in yeasts. Afterwards, it can be clearly seen a dramatic decline in the number of viable microorganisms in the subsequent sampling points. In parallel, treated rats (ZDF-T) in the *Study 1* presented a tendency for healthier (lower) levels of FBG than control group (ZDF-C). Even though there was an increase in FBG in ZDF-T during the study, that is typical of the model and the ageing process, the treatment prevented for a greater increase in FBG values, as observed in ZDF-C.

It is uncertain, at this stage, to what extend microorganisms viability is a preliminary condition for the beneficial effects of Probisan®. As shown in the bar plots, the load of total bacteria, LAB and yeasts fell to a low point. During a not much different period, the ZDF-T group kept numerically lower FBG values than ZDF-C during the whole intervention. It can thus be suggested that another Probisan®'s component(s) other than microorganisms caused the beneficial effects in the ZDF-T rats. There are further details that may support such assumption. To start, the initial load of microorganisms in Probisan® was below the minimum level estimated to provide health benefits (estimated to be $1x10^9$ CFU per serving or dose) (88). Besides, we are not aware of the *in vitro* or *in vivo* probiotic attributes of the microorganisms in Probisan®, for example their resistance to digestion (acids and bile salts), adherence to epithelial cells and mucus, formation of biofilms and colonisation, or antibacterial activity (435,436). Furthermore, even assuming that such properties are demonstrated, it is highly unlikely that their biological effects last over time in the host.

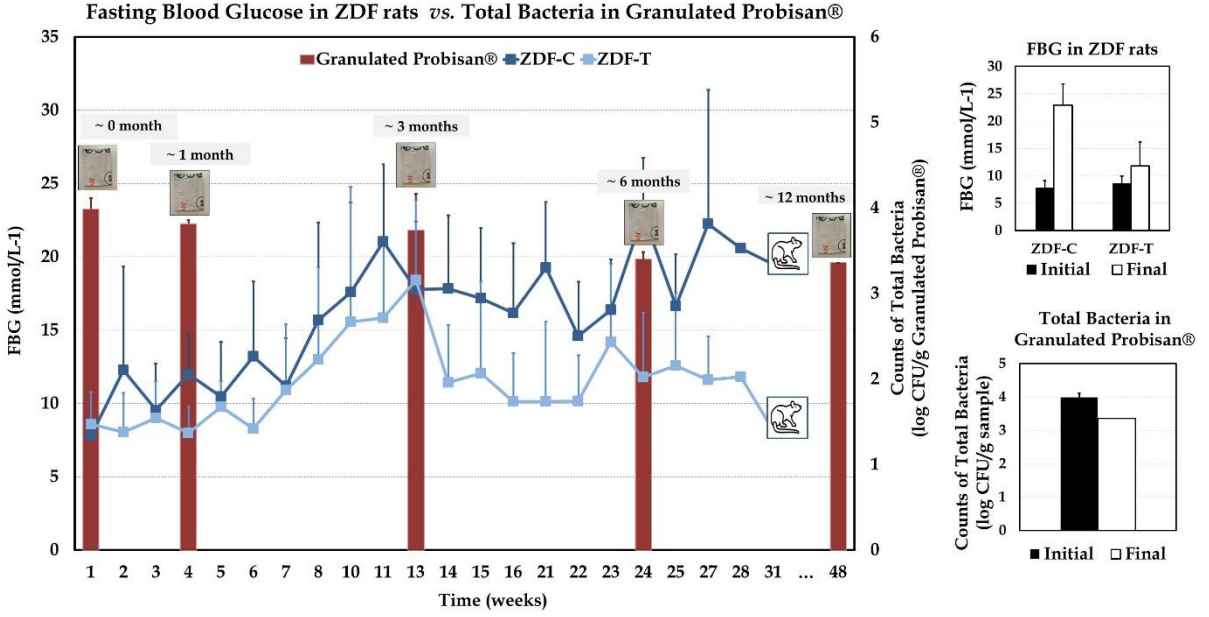

Figure 9. Schematic representation of the temporal matching between fasting blood glucose (FBG) in ZDF rats supplemented with (ZDF- T) and without Probisan® (ZDF-C) (*Study 1*) *vs.* the load of alive total bacteria in Granulated Probisan® stored at room temperature with standard packaging (RT-SP).

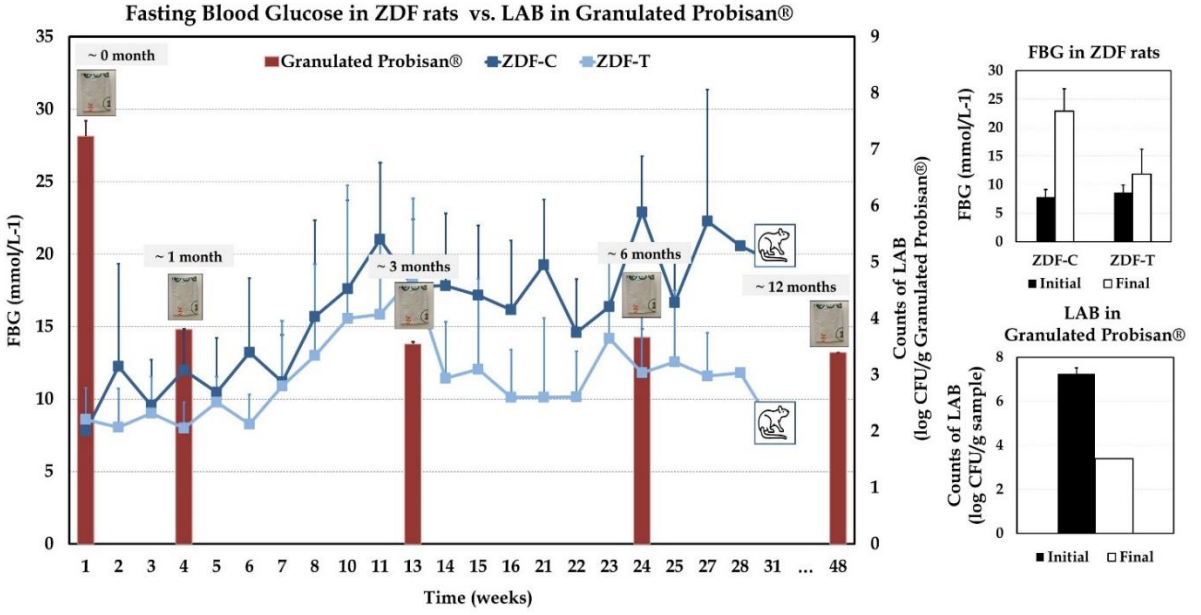

Figure 10. Schematic representation of the temporal matching between fasting blood glucose (FBG) in ZDF rats supplemented with (ZDF- T) and without Probisan® (ZDF-C) (*Study 1*) *vs.* the load of lactic acid bacteria (LAB) in Granulated Probisan in Probisan® stored at room temperature with standard packaging (RT-SP).

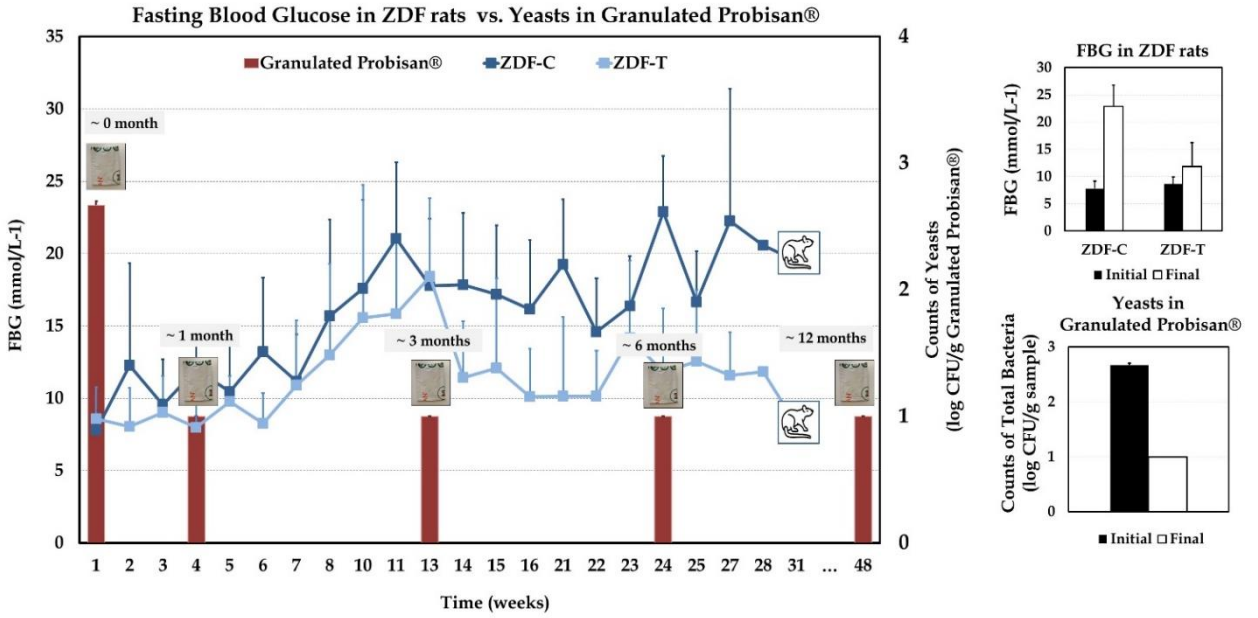

Figure 11. Schematic representation of the temporal matching between fasting blood glucose (FBG) in ZDF rats supplemented with (ZDF- T) and without Probisan® (ZDF-C) (*Study 1*) *vs.* the load of yeasts in Granulated Probisan® stored at room temperature with standard packaging (RT-SP).

In addition, there are other observations that support the hypothesis that the microbial fraction may not be essential for Probisan®'s health-promoting effects. The Probisan® surplus in the *Study* 3 was kept in our laboratory for 24 months, and the product stored at room temperature along with other variants were tested for their hypoglucemic effect (unpublished data). For this purpose, a group of BALB/c mice (n = 6) was fed with an hypercaloric diet (after 2 weeks of acclimation with standard diet) and were fed with Probisan® *ad libitum* during 5 weeks (Group 1). In parallel, another group (n = 6) was fed with Probisan® freshly produced elaborated following the standard production process (See **Figure 7** in Chapter 4) as positive control (Group 2). At the same time, a third group (n = 6) was given Probisan® elaborated without the incorporation of the fermentative inoculum to the solid substrate, as negative control (Group 3). Regarding the results, mean food intake was comparable in all the groups (3.2 ± 0.3, 3.3 ± 0.5 and 3.0 ± 1.5 g/day/mouse in Group 1, 2 and 3, respectively). Nevertheless, data from body weight and glycaemia revealed some slight differences among groups (**Supplementary Figure 1, Annex, page 284**). Overall, it was observed that the product elaborated with the microbial inoculum (Group 1 and 2) led to comparable levels of glycemia, while the product elaborated without this inoculum (Group 3) caused greater levels of glycemia. These data must be interpreted with caution, however, these results provide further support that the viability of the microbial fraction of Probisan® is not a necessary requirement to obtain beneficial effects but microorganisms must be involved in the product production.

All of the above notwithstanding, if it can be proven that the alive microorganisms in Probisan® are key responsible for its beneficial effects, more comprehensive studies on predictive microbiology, at strain level, could be performed in order to identify an effective storage that maximizes the survival of certain beneficial microorganisms.

In the light of the above mentioned, there are theoretical grounds for believing that food bioactive constituents are more likely to induce Probisan®'s health-promoting effects than microorganisms themselves. In this sense, components of the food matrix, of microbial or non-microbial origin, may be responsible for the health advantages of Probisan®. This could offer great advantages since microbial-derived bioactive molecules may be more

stable over time than alive cells, and their functionality could be preserved better through storage (33). Were this true, storage at room temperature would be enough to ensure a right preservation of the product over time.

There are theoretical grounds for believing that Probisan® contains prebiotic compounds. These include non-digestible carbohydrates and polyphenols, typically found in plants and cereals (3,437,438), some of which are expected to be present in Probisan® since it is made of soy, alfalfa and malt. Both fermentable fibers (395,399,439–442) and polyphenols (443–446) have showed favourable effects in DM and overall health in both animal and human studies, many of them mediated through the GM. Similarly, according to the producers, Probisan® is source of micronutrients like B-complex vitamins, trace elements such as zinc, manganese and selenium, amino acids like lysine and methionine, and probably it presents other biological active compounds such as n-3 fatty acids and antioxidants. All of them may have contributed to the physiological effect as well (39,61,97,447).

Regarding the microbial-derived molecules, although it has yet to be investigated, it is likely that Probisan® includes fermentation end-products such as organic acids, and microbial structures like EPS, GABA, conjugated linoleic acid or bacteriocins (26,438,448). These compounds showed to display a number of beneficial effects including prebiotic, antioxidant, antidiabetic, hypocholesterolaemic and immunomodulatory effects among others (3,449).

Particularly, it has been hypothesized that FFs exert beneficial antidiabetic effects through improvements in the antioxidant and anti-inflammatory status, and it has been suggested that phenolic compounds and other antioxidants along with GABA present in FFs are the major responsible for such effects (97).

By way of summary, it is likely that more than one component present in Probisan® contributed to protect ZDF rats from diabetes-related complications through multiple mechanisms. With the objective to clarify the mechanisms behind Probisan®'s beneficial effects, it is of primary importance to make a profound characterization of the product.

By way of summary, it is likely that more than one component present in Probisan® contributed to protect ZDF rats from diabetes-related complications. With the objective to clarify the mechanisms behind Probisan®'s beneficial effects, it is of primary importance to make a profound characterization of the product.

Influence of Probisan®'s matrix

Since we have information on the number of viable microorganisms in Probisan®-1 and Granulated Probisan®, we can compare both samples in order to obtain further information on the influence of the matrix on the microbial viability. Even though data quality is low, due to the small sample size and other limitations aboved-mentioned, we can know more about the stability of Probisan®'s microbial community.

Figure 12 graphically shows the load of viable total bacteria, LAB and yeasts in Granulated Probisan® (purple) and Micronized Probisan® (orange). As it can be seen, the initial load of total bacteria was greater in Granulated Probisan® but final values were not disparate (**Figure 12A**). Both samples suffered from a gradual decline in the number of viable bacteria. While such decline was lineal in Granulated Probisan®, Micronized Probisan® presented a slight rise following 3 months of storage, which then descends to values comparable to those in Granulated Probisan®. To continue with LAB, we can observe that in this occasion Granulated Probisan® presented a marked higher initial load (**Figure 12B**). Both products exhibited a dramatic drop following 1 month of storage, point at which values fluctuated and declined steadily until the end of the study. At that time the number of viable LAB was marginally higher in Micronized Probisan®. At baseline there was a difference of 2.55 log LAB/g product between Granulated and Micronized Probisan®, while in the subsequent sampling points the difference was much smaller. This may suggest that freshly produced Granulated Probisan® presented a different microbial composition to that in Micronized Probisan®, that include some additional bacteria species, that are absent in Micronized Probisan®, and are more susceptible to storage time. Another

explanation may be that the drying process in Granulated Probisan®, that is less intensive than that one in Micronized Probisan®, preserved better the load of microorganisms in the culture media or solid substrates utilized for Probisan® production.

Regarding yeasts **(Figure 12C)**, as with total bacteria, Micronized Probisan® had greater values. Following 1 month of storage both samples registered a sharp decrease and lost the majority of yeasts.

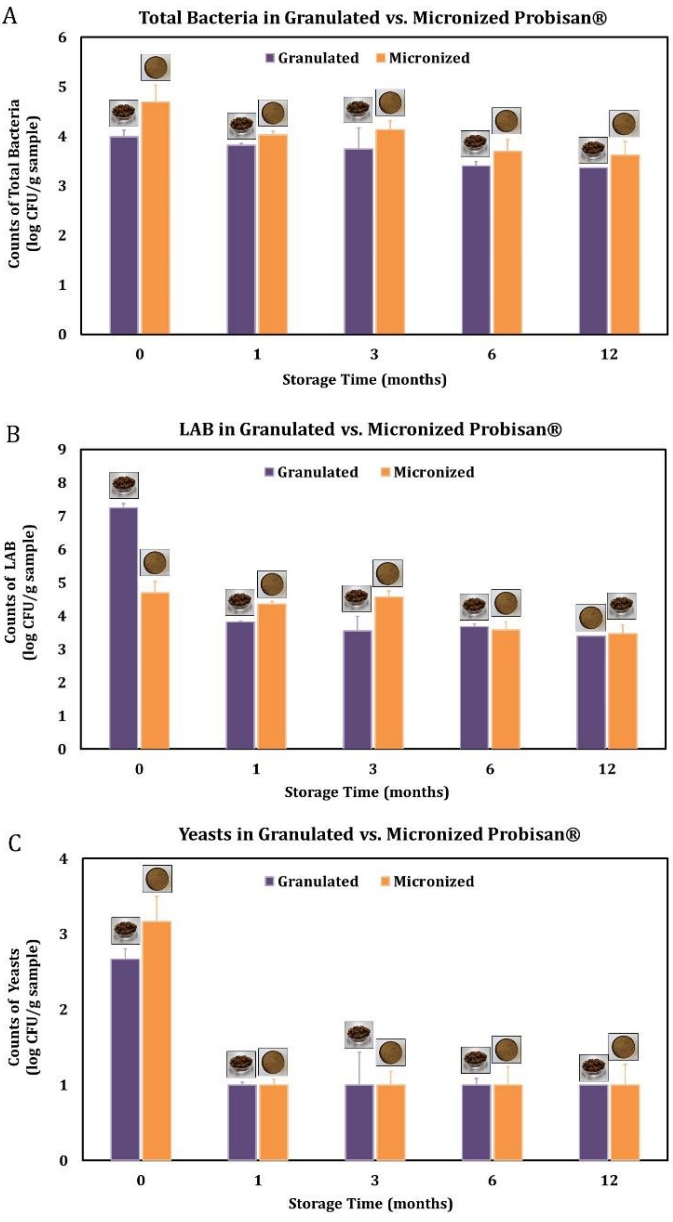

Figure 12. Dynamics of total bacteria (A), LAB (B) and yeasts (C) in Granulated and Micronized Probisan® stored at room temperature with standard packaging.

Limitations

As it has been already mentioned throughout the discussion section, we are aware that this research presents certain contrainsts. These methodological issues, many of which are due to lack of resources and time, should be acknowledged and regarded as limitations in the present thesis.

First, the general limitation of the *Study 1* is the relative small sample size. Also the need for a follow-up period should be emphasized, since it would inform about the impact of the intervention in the diabetic phenotype of the ZDF rats. A further limitation is the lack of measurements of other parameters that may clarify Probisan®'s mechanisms of action, specially a comprehensive analysis of the GM, with multi-omic technologies, and in biological samples other than faecal samples. On the other hand, the determination of biomarkers of the inflammatory processes, such as cytokines profile, would reveal new information about Probisan®'s effects.

Secondly, the *Study 2* also has a few limitations that should be considered. This work is a narrative review and this type of research presents some inherent risk of bias, particularly selection and confirmation bias. We followed a structured and organized literature search, screening and selection of studies, and extraction data. Nevertheless, our work presents some potential limitations that could be minimized by a systematic review. To illustrate, we did not elaborate an *a priori* protocol, information was not reviewed by two independent authors and we did not assess the risk of bias of the included studies.

Lastly, regarding the *Study 3*, the main limitation is the inability to analyse Probisan®'s microbial content using molecular techniques (RT-qPCR) that, without any doubt, would have provided with infinite information of the dynamics of the microbial communities in the food system. Additionally, our findings are restricted to the characteristics of the culture media and incubation conditions (time, temperature and oxygen level), and alternative growing media and culture condition would have provided with different results. In the same line, along with pH and moisture content, a real-time monitoring of the bioactive molecules (i.e., organic acids, polyphenols and other antioxidants) in Probisan®

would allow the identification of the most suitable storage conditions for such product. In a similar manner as *Study 1*, a greater number of replicates would probably reveal statistically significant differences otherwise unnoticeable. Concerning the effect of packaging mode, alternative packaging materials remain to be explored. Lastly, it is important to bear in mind that the individual packages in the *Study 3* contained 150 g of Probisan®, while regular sacks used in the company contain 25 kg. We ignore whether the product would behave differently in packages of different sizes and therefore it is unclear whether we can extrapolate to our findings.

Conclusions

The main conclusions drawn from the present thesis are summarized below:

1. The intake of Probisan® resulted in a better health status and longer life expectancy in Zucker Diabetic Fatty rats, mainly due to its effect on glycemic control and the attenuation of diabetes-derived metabolic deteriorations.

2. Probisan® supplementation did not protect against the development of type 2 diabetes, however, it caused a delay in the onset and progression of the disease by mitigating the undesirable effects of the condition.

3. Although the metagenomics analysis of faecal samples through 16S rRNA sequences did not reveal significant differences in microbial composition between treated and control animals in *Study 1*, we speculate that the intervention with Probisan® produced systemic changes that could, to some extent, be attributed to structural or functional changes of the gastrointestinal microbiota. A more exhaustive study through the application of omics sciences, such as metatranscriptomics, metaproteomics, or metametabolomics, and in alternative biological samples such as colon biopsies, could shed some light on the subject.

4. The gastrointestinal microbiota is a biological structure that strongly conditions the health status of the host, and a major factor causing disease and discomforts. Therefore, it should be considered as a therapeutic target for the management of certain diseases and complications, including the different forms of diabetes mellitus.

5. Storage, even in the short term, significantly affects the viability of Probisan® resident microorganisms, both bacteria and yeasts. On the other hand, the pH and moisture content appear to be almost unaffected by storage temperature and packaging conditions.

6. Vacuum packaging using polyethylene bags does not confer protection against loss of microbial viability in Probisan®. However, it is unknown whether other alternative packaging materials or methods could significantly increase the survival of microorganisms in the product.

7. Low storage temperatures such as refrigeration or freezing had a lower impact on the microbial viability and are therefore recommended with the purpose to preserve the highest load of alive microorganisms.

8. By bringing the results of *Study 1* and *Study 3* together, we have concluded that the beneficial effects derived from the intake of Probisan® are likely to be induced by bioactive components other than viable microorganisms. These may include microbial metabolites produced or activated during fermentation, inactivated microorganisms or dietary active compounds, all of which are known to be potential immunomodulators and health promoters. Nonetheless, fundamental knowledge about this issue is still scarce and further research is needed.

9. A profound characterization of Probisan®, including the identification of all metabolites and compounds, and the profiling, up to strain level, of its microbial ecosystem, would be extremely useful to determine its functional fraction. In the same way, such study would allow the customization of the Probisan® storage to preserve said component(s) in order to prolong the functionality of the product over time. Lastly, this new knowledge would allow the reorientation of the industrial production of Probisan® with the aim to increase its beneficial properties.

Conclusiones

Las principales conclusiones que se pueden extraen de la presente tesis son las siguientes:

1. La ingesta de Probisan® causó una mejora en el estado de salud e incremento de la esperanza de vida en las ratas Zucker Diabetic Fatty, principalmente por su efecto en el control de glucemia y la atenuación de complicaciones metabólicas derivadas de la diabetes.

2. La suplementación con Probisan® no protegió frente al desarrollo de diabetes tipo 2, sin embargo, causó un retraso en el inicio y progresión de la enfermedad al aminorar los efectos indeseables de la enfermedad.

3. Aunque el análisis metagenómico de las muestras fecales a través de secuencias de ARNr 16S no reveló diferencias significativas en la composición microbiana entre los animales tratados y los animales control en el *Estudio 1*, especulamos que la suplementación con Probisan® produjo cambios sistémicos que podrían, hasta cierto punto, atribuirse a cambios en la composición o función de la microbiota gastrointestinal. Un estudio más exhaustivo con el uso de técnicas ómicas, como la metatranscriptómica, metaproteómica o metametabolómica, en muestras biológicas alternativas como las biopsias de colon, podría arrojar algo de luz sobre el tema.

4. La microbiota gastrointestinal es un órgano que condiciona fuertemente el estado de salud del huésped y un importante factor causante de enfermedad y problemas de salud. Por lo tanto, debería considerarse como diana terapéutica para el manejo de ciertas enfermedades y complicaciones, incluyendo la diabetes mellitus.

5. La viabilidad de los microorganismos presentes en Probisan® se ve afectada de manera importante incluso a corto plazo, y se ven afectadas tanto bacterias como levaduras. Por otro lado, el pH y la humedad de la muestra no parecen verse muy afectadas por la temperatura de almacenamiento y las condiciones de envasado.

6. El envasado al vacío utilizando bolsas de polietileno no confiere protección contra la pérdida de la viabilidad microbiana en Probisan®. No obstante, se desconoce si otros materiales o métodos de envasado alternativos podrían incrementar de forma significativa la supervivencia de los microorganismos en el producto.

7. Las bajas temperaturas de almacenamiento, como la refrigeración o la congelación, tuvieron un impacto menor en la viabilidad microbiana y, por lo tanto, su uso es recomendable para preservar la mayor carga de microorganismos vivos en Probisan®.

8. Al valorar de forma conjunta los resultados del *Estudio 1* y *Estudio 3*, se puede concluir que los efectos beneficiosos observados tras la ingesta de Probisan® podrían deberse a componentes bioactivos distintos a los microorganismos viables. Estos podrían ser metabolitos microbianos producidos o activados durante la fermentación, microorganismos inactivados y otros compuestos bioactivos, todos ellos conocidos por ser potenciales inmunomoduladores y promover la salud. No obstante, nuestro conocimiento en la actualidad es limitado y es necesario llevar a cabo más investigaciones para confirmar esta hipótesis.

9. Una caracterización detallada de Probisan®, con la identificación de todos los metabolitos y compuestos, y la composición de su ecosistema microbiano hasta el nivel de cepa sería extremadamente útil para determinar su fracción funcional. Del mismo modo, dicho estudio permitiría la personalización del almacenamiento de Probisan® para preservar dicho(s) componentes(s) con el fin de prolongar la funcionalidad del producto en el tiempo. Por último, este nuevo conocimiento permitiría la reorientación de la producción industrial de Probisan® con el objetivo de intensificar sus propiedades beneficiosas.

Future Perspectives

The following sections include proposals for future lines of research to fill the gap in knowledge of Probisan® and deal with previous limitations.

Further Preclinical Studies with Probisan® or Its Derivatives

Concerning the *Study 1*, it is likely probable that a bigger sample size would have allowed us to find statistical significance in more parameters. Nevertheless, given that it was the first study with Probisan® in this animal model, and considering that this model account for a substantial cost, it seems rational to start by testing the product in a reduced sample. For coming studies, however, a bigger sample would be recommended to verify our findings in the *Study 1*.

We performed the study in the ZDF rat, a leptin receptor deficient model. Probably performing the same intervention on another murine model with no impairment in the leptin signalling may reveal different outcomes following Probisan® administration. Indeed, other authors that had tested dietary intervention in the ZDF rats suggested that the altered leptin-receptors in ZDF rats could have impeded from a number of health improvements (418). In this way, alternative preclinical models for the study of T2D such as genetic models (diabetic Goto-Kakizaki rats (115) or the Tsumara Suzuki Obese Diabetes (TSOD) mice (341)), chemically induced diabetic animals (i.e., alloxan or streptotozin-induced mice or rats) (341,450) or high fat diet-induced murine models (451), may be helpful to deepen on Probisan®'s mechanisms for increasing wellbeing and improving diabetes-related complications.

Similarly, according to *Study 2* and previously published works (377,452), performing experimental studies on T1D models would increase our understanding of Probisan®'s normoglycemic effect. As mentioned previously, we have already tested Probisan® in the NOD mice model and some favourable outcomes have been found (oral communication,

unpublished work), however, additional studies in other rodent models for T1D (i.e., Bio- breeding (BB) rat (346) or Akita mice (453)) would give us interesting data.

In the same line, it could be contemplated an experimental design that includes both sex. Besides the general interest for avoiding sex bias, the study of the sex component in the GM seem to be advisable since with both animal and human studies have insinuated that sex may influence the performance of the GM (454,455), and dietary interventions could have a sex-dependent effects on it (456).

To continue, some modifications in the experimental design may be considered by the incorporation of an additional control group with standard diet, or a negative control. Also the route of administration and the right dose should be further studied.

In addition to the foregoing, I would suggest to explore other parameters with the purpose to gain a deeper mechanistic insight. To start, it could be helpful to evaluate in a more detailed way the glucose homeostasis and metabolism by analysing serum levels of insulin, leptin, lactate or glycerol (457), the percentage of glycosylated haemoglobin (458), the expression of genes implicated in insulin signalling (i.e., AKT kinase, insulin receptor, adipose or muscle glucose transporter 4) (459) or the energy expenditure (457,460). Secondly, it can also be explored the inflammatory status by checking the activity of enzymes with antioxidative properties (408) the expression of genes involved in the inflammatory response (459), the lipid peroxidation (408), a panel of circulating cytokines (376,457) or levels of C-reactive protein (461), for instance.

Being that diabetic subjects frequently present an altered gut barrier function (462,463) and impaired glucose transporters in the gut (464), the state of the intestinal integrity may release important health information as well. For example, the barrier function could be studied in a more direct way, by checking the *in vivo* permeability (465) and performing assays of intestinal permeability following the intake of sugar solutions (457,466)), or using surrogates measures associated to the barrier function such as serum (466) or faecal zonulin levels (467), and serum LPS (466) or LPS binding protein (468). Additional parameters of intestinal health are markers of intestinal inflammation (i.e., calprotectin,

secretory IgA, or faecal human beta-defensin-2) (376,469), markers of epithelial cell turn over (460) and levels of gut hormones such as GLP-1 (223,457), GLP-2 (465) glucose-dependent insulinotropic peptide (439) or peptide YY (317), that exert very relevant functions.

The study of markers of microbial fermentation in cecal content (i.e., empty cecal weight, level of SCFA or pH values) (418) or faecal samples (i.e., pH values, profile of SCFA or bile acids, moisture) (293,470) or the evaluation of stool frequency, volumen and consistency (471,472) are also widely used for studying the impact of dietary intervention on the GM's functioning.

Equally important, and as previously suggested in the discussion of the thesis, a more comprehensive analysis of the intestinal microbiota would be greatly useful in resolving some uncertainties. In this way, a multi-omic approach performing a functional metagenomics study along with the use of other omics technologies such as metametabolomics, metaproteomics or metatranscriptomics (473), would reveal if the supplementation with Probisan® induces or not alterations in the microbial performance.

Even though animal studies like *Study 1* are exceedingly helpful for the generation of knowledge prior to human studies (230), it is always challenging to translate discoveries in the laboratory to the clinical setting. Besides, the small simple size reduces the external validity of our findings. To date, whether findings from the *Study 1* translate into clinical benefits in humans remains unclear. It is thus necessary to deepen our understanding of the mechanisms behind Probisan® effects and a reasonable approach to tackle this issue could be performing further preclinical studies. Nonetheless, besides much research needs to be done, the application of Probisan® in humans looks promising as a nature-based therapeutic strategy for T2D and probably other diseases of the century.

Moreover, there is a niche for Probisan® in the field of functional food. In recent years, the development of novel food products has drawn the attention of researchers since the intake of functional foods such as FFs (3,37) or foods enriched with specific components (474,475) seemed to be an effective way to improve health. Much research has been performed with the aim to increase the quality of foods. To illustrate, many studies aimed

to fortify bakery products with additional sources of dietary fiber (476,477) or protein (478). For example, the incorporation of prebiotics (inulin-type fructans) in a gluten-free bread elaborated with rice flour enhanced the nutritional quality of the product and reduced glycemic response (measured with *in vitro* predicted glycemic response and *in vivo* analysis with subjects) (479). What is more, the authors found a greater consumer acceptability of the bread fortified with prebiotics. In a 2017 study, pasta partially elaborated with quinoa flour fermented by LAB presented an improved nutritional characteristics (protein quality and digestibility, greater total phenols, antioxidant activity and fiber content) and had a reduced predicted glycemic index in comparison to the control product (480). In this way, Probisan® could be incorporated to a product already marketed either as an additional ingredient in the food formulation or replacing (totally or partially) another ingredient, with the purpose to enhance the nutritional value of the final product. Moreover, in a small pilot study completed during the research internship in South Dakota State University with Prof. Maristela Rovai and Prof. Padmanaban Krishnan, we developed a bread with different replacement of flour with Probisan® (See **Supplementary Figure 2, Annex, page 285**). We completed a number of experiments to evaluate physicochemical and texture characteristics of the experimental loaves and consumer acceptability was assessed with a consumer panel (unpublished work). Preliminary results suggest that there is a potential application of Probisan® for the development of functional foods.

Alternatively, Probisan® could be used to develop a nutraceutical, that refers to products, most of them with pharmaceutical forms (6,449), that offer benefits beyond the nutritional value and are used to improve health and reduce disease (6,100).

A Comprehensive Description of Probisan®

To date, there are many remaining gaps in our knowledge of the mechanisms underlying Probisan® effects and the picture is thus incomplete. Hopefully, still many experiments can be performed to unveil the components whereby Probisan® may alleviate the diabetic phenotype and improve health.

There is a constellation of factors in Probisan® that could explain its beneficial effects. Firstly, it provides refuge to a myriad of microorganisms, some of them alive. Secondly, Probisan® includes an array of microbial-derived compounds. Some of them are generated during its industrial processing, while others occur naturally in its raw materials. Moreover, owing to the plant-origin of Probisan®'s ingredients, its raw materials are likely to be a good source of prebiotic compounds and phytonutrients.

Overall, the current understanding of Probisan®'s matrix suggests that all the compounds and molecules that merge in it collectively exert favourable effects on the consumer. A number of analysis can be useful to gain more in-depth knowledge about the exact mechanisms. To illustrate, an exhaustive characterization of the microbial population in Probisan® (not only bacteria but also other microorganisms such as yeasts and molds) has not been conducted in depth. Once identified all the microorganisms present in Probisan®, novel strains or previously unidentified species could be tested to analyse their probiotic properties (i.e., tolerance to gastric conditions and surface properties such as aggregation and hydrophobicity) (432,435,436), could be registered in a microbial strain collection and be characterized. Even if they do not display probiotic properties, microbes-based therapies with transient food microorganisms may be able to exert beneficial effects on the consumer.

To continue, a detailed characterization on the biochemical composition of Probisan® is largely lacking. It could be particularly interesting if our assumptions about the little, if any, effect of alive microorganisms, turns out to be true. Such characterization could be done by determining total, soluble and insoluble dietary fiber (481), profiling the composition of organic acids (i.e., acetic, succinic or lactic acid), or identifying prebiotic compounds (i.e., β-glucan galactan, fructan) (95,482). Besides, it could be evaluated the potential *in vivo* antioxidant activity or the abundance of bioactive compounds with antioxidant activity,

such as phenolic compounds (458,483–485), bioactive peptides, vitamins or EPS (486). It is worth pointing out that, although we could measure the amount of antioxidant compounds or the antioxidant property in a food matrix, this does not necessarily translate into the same antioxidant activity in the host. Both the resistance to the digestion and the bioavailability in the tissue will ultimately determine the antioxidant property for a given food (486).

Further physiologically active components that offer health benefits are β-glucans (165) or microbial EPS (47,486). Also of note, metabolomics studies profiling the set of fatty acids, amino acids, saccharides and vitamins in our product would be helpful (19,487,488).

Once identified the major elements in Probisan®, an important issue to resolve for future studies is which(s) of these components translate into meaningful health benefits (488). Special attention should be paid to polyphenols, such as phenolic acid, flavonoid, tannin, or lignin (486), that render an invaluable health benefit to consumers, including antidiabetic properties (489,490). Microbial-derived compounds such as EPS (394) and biosurfactant (491) also demonstrated an antidiabetic effect or protection against diabetes complications in murine models. Lastly, it cannot be disregarded the beneficial effect of prebiotics in DM (399,439,441).

Further Research into Probisan®

As with so many research projects, in the *Study 3* it was not possible to evaluate and explore all the parameters that would be interesting for having an extensive knowledge of Probisan® and derive full benefit from it. We only explored the final stage of food production, however, much research can be done in previous phases such as food formulation and processing.

It is well-known that food processing may influence the nutritional profile, (i.e., content in vitamins and antioxidant compounds) (486,492) and functionality of bioactive compounds (165). In this light, the processing process could thus be improved to obtain a final product with greater nutritional properties.

To start with food formulation, the incorporation of protectants or growth promoters can positively affect the microbial performance and viability (43,84). This can be illustrated by the addition of prebiotics into the microbial cultures, that causes an increase in the amount of vitamins in the final product. This strategy can be helpful to counteract the negative effect of storage time on vitamins content (492). Similarly, an enrichment in micronutrients has been observed following the incorporation of ascorbic acid or Na- FeEDTA in fermented beverages (19). Increasing the content of compounds with antioxidant properties is also a good strategy for enlarging Probisan®'s shelf life. This could be achieved by selecting a suitable starter culture according to the matrix, accompanied by a readjustment of the fermentation condition (486).

Further, other details such as the selection of an operative microbial culture (considering the interactions within species and strains and dose), the control of the generation of secondary metabolites, and the monitoring of the physicochemical environment in the fermenter (i.e., pH and oxygen content) may be helpful for the enhancement and improvement of the production process (19,33,43).

Besides modifications in food formulation, food processing could also be refined with changes in the technological processes. To illustrate, it is recommendable to take into account the physiologic state of bacteria during growth, since they are more sensible when they are in the logarithmic phase in comparison to the stationary phase (27). This needs to be taken into account when proceeding to the next stage of processing.

Further, the selection of temperature and time of the different processes cannot be disregarded (19,43). This could be particularly interesting for the paraprobiotic or postbiotic fraction in Probisan® (65), since different thermal treatments have a distinct impact on the biological effects of inactivated or non-viable microorganisms (64,124). Moreover, food processing may influence the effects of food on the gut microbiota and enhance their health-promoting effects. A very interesting research was performed to evaluate the influence of whole-grain barley and barley malt on the microbial ecology of rats fed with a high fat diet (68). The intervention induced significant changes on GM composition and SCFA, and authors suggest that changes occurring during the malting

process (including differences in dietary fiber composition, molecular weight, fermentability, protein digestibility and iron availability) may be major responsible for such differences.

Other strategies can be established to increase the amount of EPS in food. Besides they have a major application in the food industry, acting as emulsion stabilisers and texturisers (51), they also display some health benefits, including prebiotic effect among others (33,47). We should not lose sight of the fact that EPS have a functional role for microorganisms. In this sense, EPS could exert a protective effect for the alive microorganisms in a food system, be metabolized by adjacent bacteria (48) and attenuate microorganisms sensibility during food processing or storage (40,47,52). Although the production of huge amount of EPS involves many constraints on the industrial manufacturing, some refinements in the design of the microbial starter culture and the culture media could result in greater levels of bioactive compounds, EPS among them (33,47).

Lastly, methods to increase the viability of microorganisms during processing has been widely studied. Those comprise both food formulation (selection of starter and adjuvant cultures, genetic manipulation,) and food processing (130,154,163,493), as well as the use of specific practices to protect microbial integrity, such as the use of ME (172,173) or the incorporation of protectants (i.e., cryoprotectants, whey protein, lactose) (43,493).

List of Conference Papers

Oral Presentations

1. **Cabello-Olmo M (communicator)**, Araña M, Torre P, Sainz N, Moreno-Aliaga MJ, Sanzol G, Díaz JV, Encío I, Barajas M. Oral supplementation with a synbiotic product (Probisan®) and its impact on the development of type II diabetes mellitus. XI Meeting Red Española de Bacterias Lácticas (Redbal) (Spanish Network of Lactic Acid Bacteria). Gijón Convention Bureau, Gijón, Spain. June 28-30, 2017.

2. **Cabello-Olmo M (communicator),** Araña M, Torre P, Sainz N, Moreno-Aliaga MJ, Sanzol G, Díaz JV, Encío I, Barajas M. Un pienso fermentado con carácter postbiótico (Probisan®) retrasa la aparición de diabetes tipo 1 que desarrolla el ratón NOD. XII Meeting Red Española de Bacterias Lácticas (Redbal) (Spanish Network of Lactic Acid Bacteria). Instituto de la Grasa - Consejo Superior de Investigaciones Científicas, Seville, Spain. May 17-18, 2018.

Poster Presentations

1. **Cabello-Olmo M (communicator),** Araña M, Sáinz M, Moreno-Aliaga MJ, Sanzol G, Encío I, Barajas M. Efectividad de un simbiótico (Probisan®) en el desarrollo y prevención de diabetes mellitus tipo II / Effectiveness of a synbiotic (Probisan®) in the development and prevention of type II diabetes mellitus. Communication presented twice: VIII Workshop Spanish Society of Probiotics and Prebiotics (SEPyP). Faculty of Medicine, University of Santiago de Compostela, Santiago de Compostela, Spain; February 23-24, 2017; and II Conference of Obesity and Metabolic Complications, XIII National Conference of the Spanish Society for the Study of Obesity (SEEDO) and XIX National Conference of the Spanish Society of Obesity Surgery (SECO). Hotel Barceló Sevilla Renacimiento, Seville, Spain. March 15-17, 2017.

2. **Cabello-Olmo M (communicator)**, Araña M, JV Díaz, Sanzol G, Encío I, Barajas M Effectiveness of Probisan® in the prevention of Non-Obese Diabetic mouse type 1 diabetes mellitus. IX Workshop Spanish Society of Probiotics and Prebiotics (SEPyP). Zaragoza Convention Bureau, Zaragoza, Spain. February 15-16, 2018.

3. **Cabello-Olmo M (communicator),** Araña M, Oneca M, Sainz N, Moreno-Aliaga MJ, Encío I, Barajas M. A Non-diary Fermented Products Attenuates Hyperglycemia and Improves Metabolic Parameters in Zucker Diabetic Fatty (ZDF) rats. VIII Congreso Iberoamericano de Nutrición. Baluarte Convention Centre, Pamplona, Spain. July 3-5, 2019.

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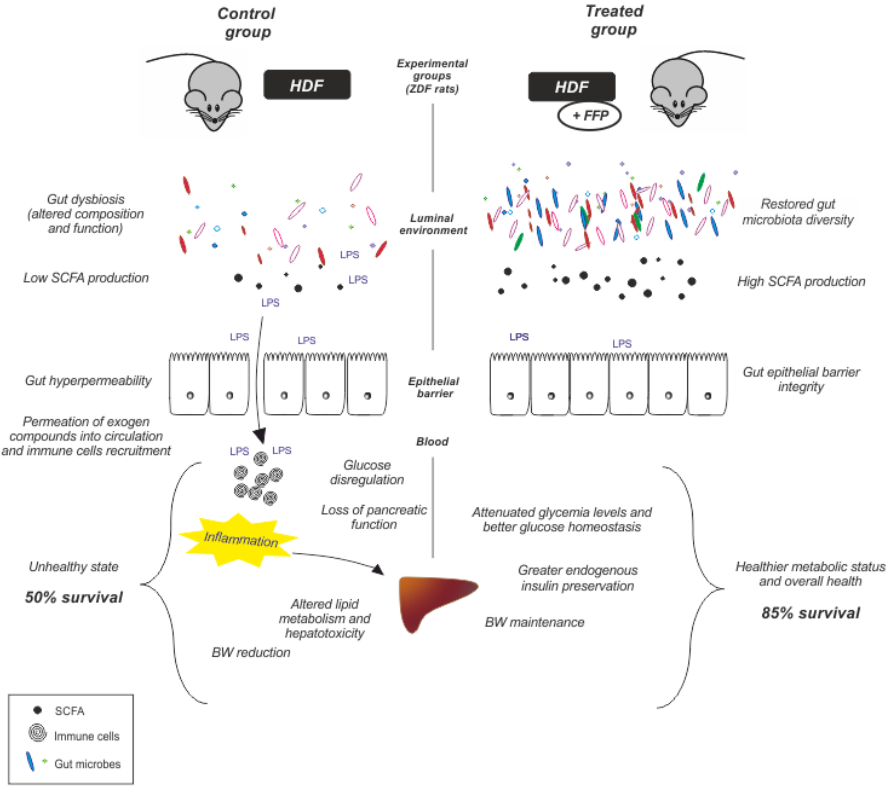
Annex

Graphical Abstract Study 1



Graphical Abstract. The FFP had the potential to improve glucose homeostais and prevent from metabolic alterations in T2D rats throught the restoration of gut health and the maintenance of normal organ functions.

Supplementary Materials Study 1

Table S1. Culturable and Viable Counts Determined in the FFP.

Media and conditions	Target microorganisms	Viable counts (UFC/g)
Trypticase soy agar, 37ºC for 24-48h, aerobiosis	Total bacteria	2.0×10^5
Man, Rogosa and Sharpe agar, 37ºC for 24-48h, anaerobiosis	Lactobacilli	4.6×10^7
Potato dextrose agar, 25ºC for 2-4 days, aerobiosis	Yeast and fungi	1.0×10^5

Table S2. Composition of FFP and ENVIGO TD.06416 Hypercaloric Diet (per 100 g of product).

Components	FFP	TD.06416
Calories (kcal)	467.6	510.0
Fats (%)	2.4	10.2
Proteins (%) (Nx6.25)	44.5	20.0
Carbohydrates (%)	53.1	69.8

Table S3. Body Fat Weight For Rats in C and T Groups. No statistical significant differences between groups were found in the weight of the different fats. The results are expressed as the mean value ± SD. g: grams, %: percentage.

	C group	T group
Body fat (g)	147.3 ± 42.0	163.6 ± 77.5
Body fat BW-1 (%)	31.7 ± 3.5	34.0 ± 8.4
Retroperitoneal fat (g)	16.3 ± 6.5	28.5 ± 16.1
Epididymal (g)	9.5 ± 1.7	13.5 ± 4.0
Mesenteric fat (g)	4.6 ± 0.7	5.1 ± 2.1
Subcutaneous fat (g)	15.5 ± 5.3	24.7 ± 12.1
Brown fat (g)	0.4 ± 0.2	0.9 ± 0.4

Table S4. Follow-up of Lipid Profile in ZDF Rats. Means in the same column with different letters A, B or C and the same row with different letters a or b differ significantly ($p< 0.05$). n=8 animals/group. Data are expressed as mean ± SD. TC: serum total cholesterol; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; TG: triglycerides.

	Time (month)	C group	T group
TC (mg dL^{-1})	0	155.3 ± 11.7^{Aa}	143.3 ± 10.8^{Aa}
	2	395.1 ± 55.6^{BCa}	353.9 ± 95.2^{Ba}
	4	380.5 ± 35.3^{Ba}	342.1 ± 77.6^{Ba}
	7	478.4 ± 61.2^{Ca}	424.7 ± 147.0^{Ba}
LDL-C (mmol L^{-1})	0	0.5 ± 0.1^{Aa}	0.3 ± 0.1^{Ab}
	2	2.2 ± 0.6^{Ba}	1.5 ± 1.2^{Ba}
	4	2.4 ± 0.5^{Ba}	2.1 ± 0.9^{Ba}
	7	3.4 ± 0.8^{Ca}	2.9 ± 2.0^{Ba}
HDL-C (mmol L^{-1})	0	2.8 ± 0.5^{Aa}	2.5 ± 0.4^{Aa}
	2	6.5 ± 0.7^{Ba}	5.4 ± 2.0^{Ba}
	4	6.5 ± 0.8^{Ba}	6.5 ± 1.3^{Ba}
	7	6.3 ± 2.1^{Ba}	7.2 ± 2.1^{Ba}
TG (mg dL^{-1})	0	223.4 ± 44.8^{Aa}	311.5 ± 95.7^{Ab}
	2	530.1 ± 129.0^{Ba}	585.9 ± 135.3^{Ba}
	4	531.3 ± 162.4^{Ba}	481.9 ± 90.2^{Ba}
	7	791.0 ± 267.4^{Ba}	452.7 ± 148^{ABb}

Table S5. List of Bacteria Species and OTUs Which Were Identified Exclusively in the C Group.

OTU1562|NN=Clostridium_glycolicum_AY007244|D=96.5
Clostridium_celatum
OTU1634|NN=Blautia_stercoris_HM626177|D=94.7
OTU407|NN=Soleaferrea_massiliensis_JX101688|D=87.4
Clostridium_methylpentosum
OTU270|NN=Dorea_massiliensis_JX101687|D=94.1
OTU1336|NN=Clostridium_methylpentosum_Y18181|D=91.5_2
OTU997|NN=Catabacter_hongkongensis_AB671763|D=82.9
OTU566|NN=Clostridium_hathewayi_EF408243|D=97
Abiotrophia_para-adiacens
Citrobacter_werkmanii
Staphylococcus_schleiferi
OTU1353|NN=Clostridium_citroniae_DQ279737|D=92.5
OTU1178|NN=Papillibacter_cinnamivorans_AF167711|D=89.4
OTU307|NN=Sphingobacterium_multivorum_KF535155.1|D=91.3
OTU1282|NN=Roseburia_faecis_AY804149|D=92.8
Abiotrophia_defectiva
OTU1739|NN=Enterobacter_cancerogenus_JN644583|D=96.9
OTU1347|NN=Eubacterium_dolichum_AB649277|D=77.1
OTU1201|NN=Blautia_glucerasea_AB588023|D=94.1
Propionibacterium_acidipropionici
OTU155|NN=Sphingomonas_panni_AJ575818|D=96.7
OTU1006|NN=Eubacterium_rectale_AY804151|D=93.3
Candidatus Stoquefichus_massiliensis
Blastococcus_massiliensis
OTU1222|NN=Clostridium_sporosphaeroides_CLORR16SAD|D=91.2

Table S6. List of Bacteria Species and OTUs Which Were Identified Exclusively in the T Group (*Part 1*).

OTU42|NN=Ruminococcus_gauvreauii_EF529620|D=88.4
OTU891|NN=Blautia_coccoides_EF025906|D=93.1
Desulfovibrio_fairfieldensis
OTU523|NN=Barnesiella_intestinihominis_AB370251|D=82_2
Neisseria_macacae
OTU744|NN=Ruminococcus_callidus_X85100|D=91.1
OTU1427|NN=Clostridium_clariflavum_NR_102987.1|D=82.3
OTU523|NN=Barnesiella_intestinihominis_AB370251|D=82
OTU1597|NN=Eubacterium_sulci_AJ006963|D=89.4
Alistipes_finegoldii
OTU1461|NN=Desulfitobacterium_frappieri_DFU40078|D=84.5
Neisseria_elongata
Lactococcus_garvieae
OTU1319|NN=Roseburia_intestinalis_AB661435|D=88.7_2
OTU1391|NN=Clostridium_clariflavum_NR_102987.1|D=84
OTU1704|NN=Clostridium_sporosphaeroides_CLORR16SAD|D=91.6
OTU254|NN=Stenotrophomonas_rhizophila_AB539813|D=96.6
OTU661|NN=Blautia_wexlerae_EF036467|D=94.9
Pediococcus_damnosus
Pseudomonas_oleovorans
OTU1116|NN=Clostridium_hathewayi_EF408243|D=93.2
Blautia_torques
OTU167|NN=Soleaferrea_massiliensis_JX101688|D=88.5
OTU570|NN=Ruminococcus_gauvreauii_EF529620|D=91.2_4
OTU82|NN=Clostridium_lactatifermentans_AY033434|D=93.1
OTU930|NN=Brevibacillus_agri_AY319301.1|D=78.3
Brevundimonas_vesicularis
Bacillus_megaterium
Microbacterium_phyllosphaerae
Lactococcus_lactis
OTU184|NN=Ochrobactrum_anthropi_KC845230|D=79
OTU446|NN=Brachybacterium_paraconglomeratum_EU660345|D=96.1
Stenotrophomonas_maltophilia
OTU1064|NN=Leuconostoc_gelidum_KF577567|D=96.8
Lactobacillus_paracasei
Butyrivibrio_crossotus
Catabacter_hongkongensis
OTU1487|NN=Melainabacter_A1|D=96.1
OTU152|NN=Oscillibacter_valericigenes_AB238598|D=94.3
OTU1546|NN=Soleaferrea_massiliensis_JX101688|D=87.8
OTU175|NN=Roseburia_hominis_AB661434|D=94.3
OTU287|NN=Coprococcus_catus_AB361624|D=96.2
OTU429|NN=Clostridium_methylpentosum_Y18181|D=81.9_2
OTU644|NN=Dorea_massiliensis_JX101687|D=88.1
OTU821|NN=Massilia_aurea_AM231588|D=96.5
OTU823|NN=Clostridium_bartlettii_AY438672|D=96.4
OTU826|NN=Lactobacillus_vaginalis_GQ422709|D=94
Serratia_marcescens
Streptococcus_parauberis
Tyzzerella_lactatifermentans

Table S6. List of Bacteria Species and OTUs Which Were Identified Exclusively in the T Group (*Part 2*).

OTU1073|NN=Barnesiella_intestinihominis_AB370251|D=87.1
Bacteroides_stercoris
Sutterella_stercoricanis
Lysinibacillus_massiliensis
Proteus_mirabilis
OTU975|NN=Bacteroides_fluxus_AB547642|D=96.1
OTU1478|NN=Ruminococcus_flavefaciens_AY349157|D=94
OTU69|NN=Oscillibacter_valericigenes_AB238598|D=94.8
OTU883|NN=Clostridium_lactatifermentans_AY033434|D=90.6
Tyzzerella_lactatifermentans
OTU193|NN=Barnesiella_intestinihominis_AB370251|D=85.1
OTU60|NN=Ruminococcus_bromii_DQ882649|D=89.6
OTU217|NN=Gemmiger_formicilis_GU562446|D=96.5
OTU521|NN=Papillibacter_cinnamivorans_AF167711|D=89.6
OTU1242|NN=Eubacterium_cellulosolvens_AY178842|D=93.3
OTU815|NN=Pseudomonas_monteilii_GQ284481|D=96.2
OTU478|NN=Butyricicoccus_pullicaecorum_EU410376|D=91.9
OTU267|NN=Parasutterella_excrementihominis_AB370250|D=92.5
OTU779|NN=Lysinibacillus_sphaericus_AJ311894|D=94.4
OTU1337|NN=Desulfovibrio_desulfuricans_DVURRDA|D=89
OTU1101|NN=Clostridium_lactatifermentans_AY033434|D=94
OTU1735|NN=Pseudomonas_monteilii_GQ284481|D=96.6
OTU1212|NN=Ruminococcus_lactaris_NR_027579.1|D=87.5
OTU1381|NN=Pseudomonas_fluorescens_KJ161327|D=96.2
OTU655|NN=Clostridium_asparagiforme_AJ582080|D=89.7_2
OTU45|NN=Soleaferrea_massiliensis_JX101688|D=88.7
OTU814|NN=Anoxystipes_fissicatena_NR_104800.1|D=92.5_2
Odoribacter_laneus
Lachnoclostridium_indolis
OTU1508|NN=Eubacterium_rectale_AY804151|D=94.1
OTU328|NN=Eubacterium_ventriosum_EUBRRDAB|D=94.6
Bifidobacterium_longum
OTU522|NN=Melainabacter_A1|D=92.9
OTU1330|NN=Clostridium_lactatifermentans_AY033434|D=90.5
OTU1119|NN=Soleaferrea_massiliensis_JX101688|D=89.3
Actinomyces_canis
OTU1475|NN=Clostridium_clariflavum_NR_102987.1|D=83
OTU814|NN=Anoxystipes_fissicatena_NR_104800.1|D=92.5_3
OTU1570|NN=Clostridium_clariflavum_NR_102987.1|D=79.3
OTU1722|NN=Clostridium_clariflavum_NR_102987.1|D=83.6
OTU239|NN=Adlercreutzia_equolifaciens_AB306660|D=95
OTU444|NN=Ruminococcus_albus_AY445596|D=91.1
OTU315|NN=Clostridium_clostridioforme_AY169422|D=91.7
OTU722|NN=Oscillibacter_valericigenes_AB238598|D=91.6
OTU944|NN=Intestinimonas_butyriciproducens_JX101685.1|D=91.5
OTU1424|NN=Dorea_formicigenerans_EUBRRDP|D=96.7
OTU1108|NN=Clostridium_bolteae_AJ508452|D=92.8
OTU1598|NN=Oscillospira[Pseudoflavonifractor]_capillosus_AY136666|D=90.1
OTU355|NN=Clostridium_lactatifermentans_AY033434|D=90.6
Psychrobacter_arenosus
OTU1115|NN=Coprococcus_comes_EF031542|D=95

Figure S1. Image of the FFP. The FFP is presented as a dry granulated product, with an average particle size ranging from 4 to 12 mm with a moisture content of 12.8% and a pH of 4.4. FFP: fermented food product.

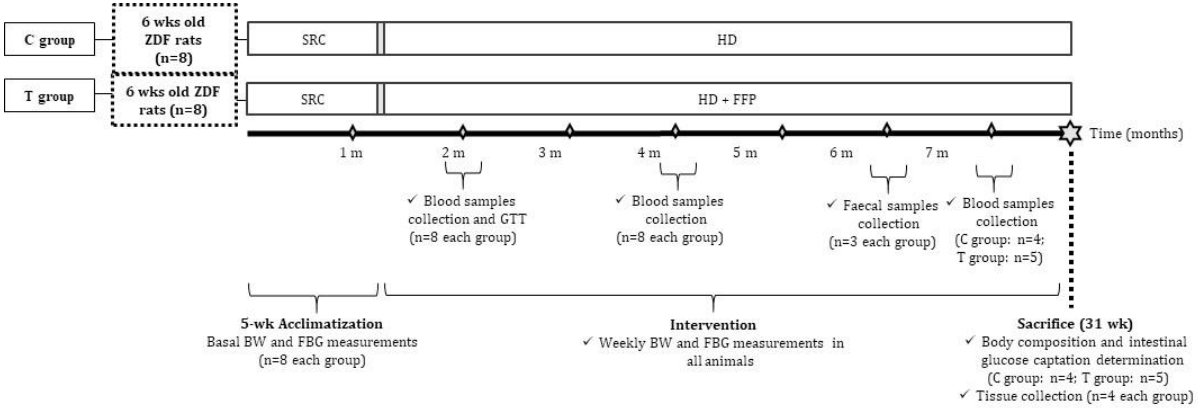


Figure S2. Experimental Design. BW: body weight; FBG: fasting blood glucose; FFP: fermented food product; GTT: glucose tolerance test; HD: hypercaloric diet; SRC: standard rodent chow.

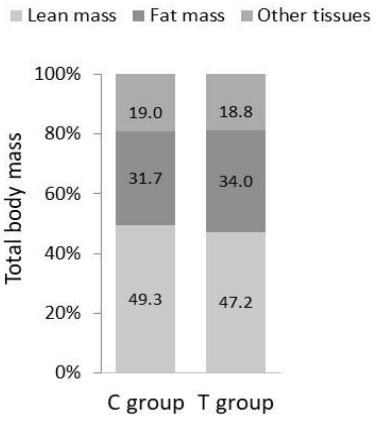


Figure S3. Body Composition at the Time of Sacrifice Measured by Nuclear Magnetic Resonance (NMR). The results are expressed as relative contribution of fat mass, lean mass and other tissues No statistical significant differences were observed between C and T group in lean mass (49.3 ± 3.9 *vs.* 47.2 ± 7.7 % respectively; $p = 0.641$), fat mass (31.7 ± 3.5 versus 34.0 ± 8.4 % respectively; $p = 0.630$) and other tissues (19.0 ± 5.6 versus 18.8 ± 3.5 % respectively; $p = 0.947$).

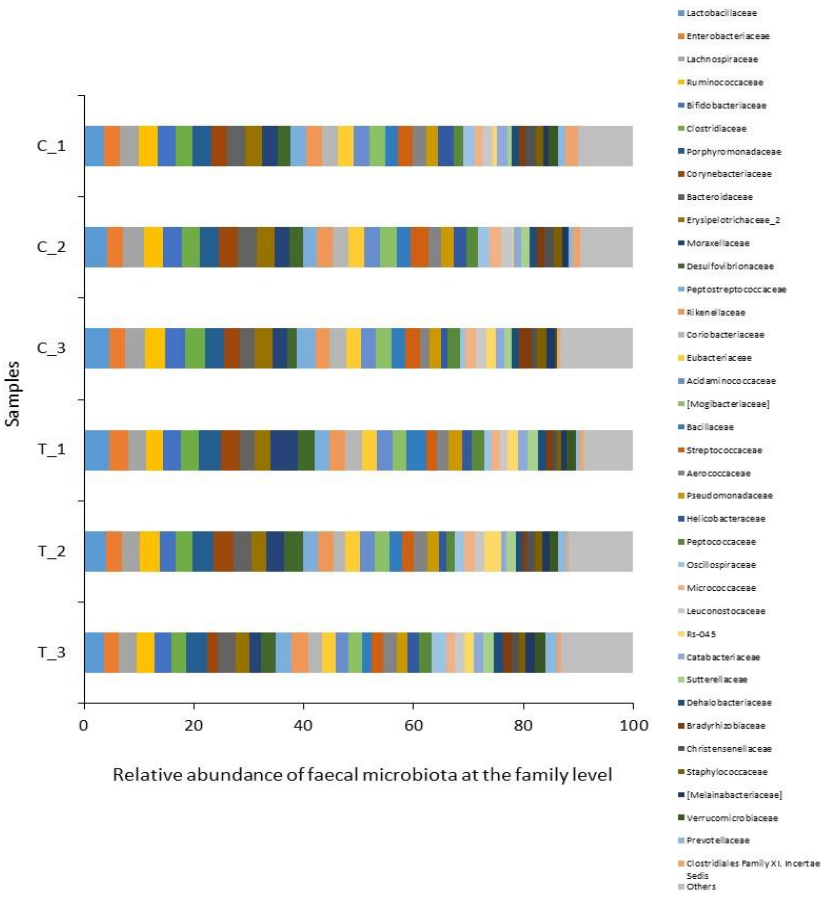
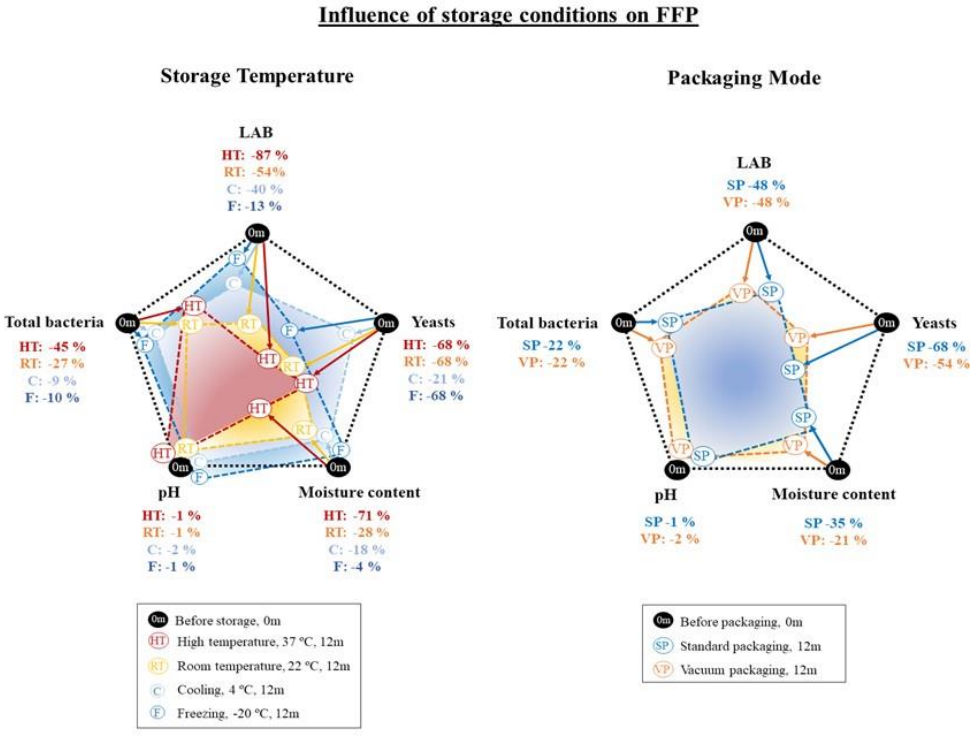


Figure S4. Relative Abundance of Faecal Microbiota at the Family Level. C_1, C_2 and C_3 are samples of C group and T_1, T_2 and T_3 are samples of T group.

Graphical Abstract Study 3



Graphical Abstract. Comparative effect of storage temperature and packaging mode following 12 months storage (experimental conditions vs. reference condition; room temperature and standard packaging for storage temperature and packaging mode, respectively). The environmental temperature showed a greater influence on the studied parameters, especially on the microbiological profile. While lower temperatures (F and C) evidenced a tendency for maintaining initial values in comparison to the reference, high temperature (HT) led to a neutral or negative influence. Regarding the packaging mode, it barely affected the studied parameters. The effect of vacuum was more noticeable in moisture content and yeasts, which suffered from a slight positive effect. FFP: fermented food product; LAB: lactic acid bacteria.

Supplementary Materials Study 3

Figure S1. Sample of Fermented Food Product (FFP).

Figure S2. Different Packaging Modes Used in the Study. Standard packaging (A) and vacuum packaging (B).

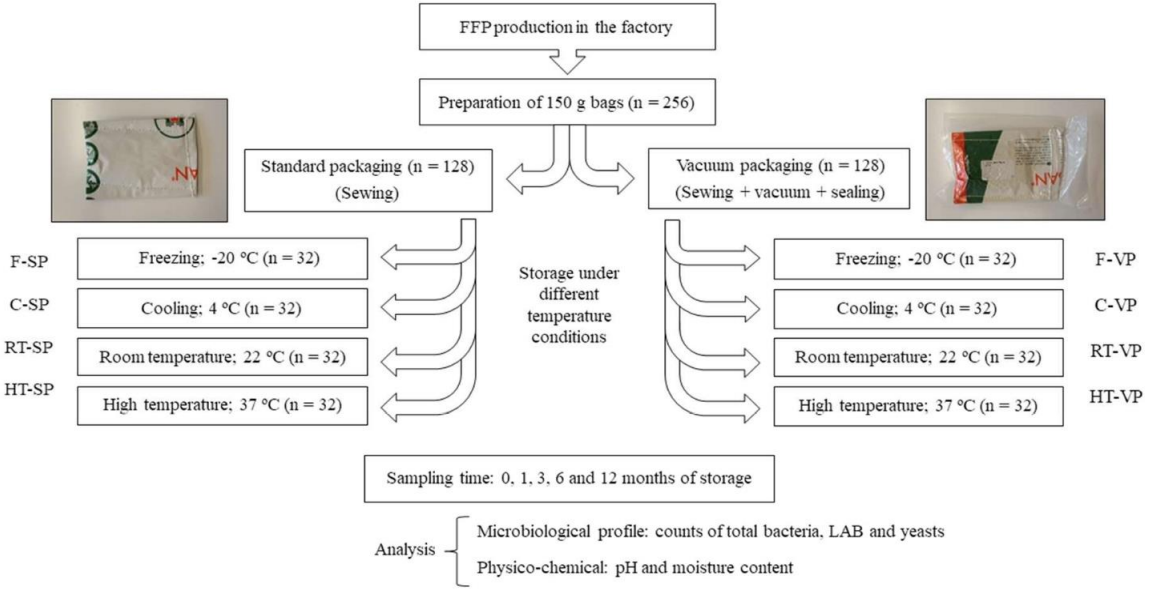
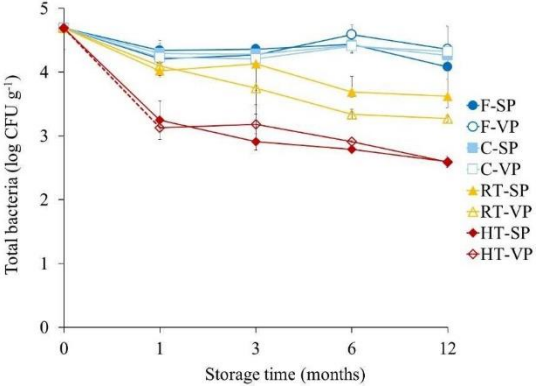
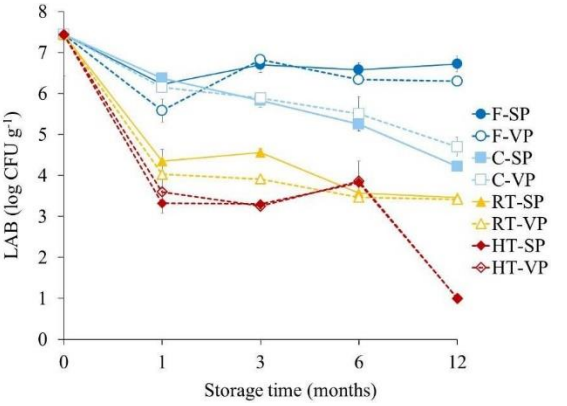


Figure S3. Experimental Design Scheme. FFP: fermented food product; LAB: lactic acid bacteria; F-SP: freezing standard packaging; C-SP: cooling standard packaging; RT-SP: room temperature standard packaging; HT-SP: high temperature standard packaging; C- VP: cooling vacuum packaging; RT-VP: room temperature vacuum packaging; HT-VP: high temperature vacuum packaging.



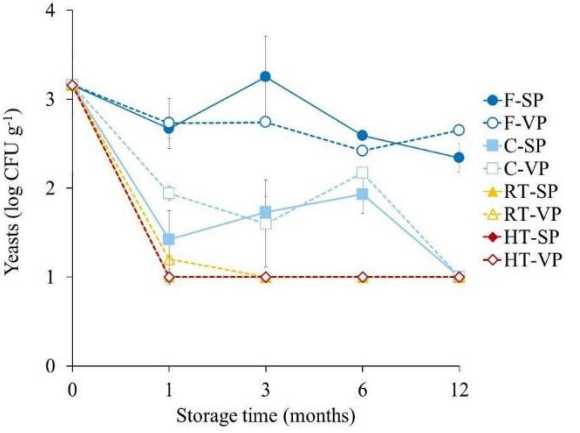
Sample Code	Temperature	Packaging	Storage Time (months)				
			0	1	3	6	12
F-SP	-20 ºC	Standard	4.69 ± 0.34	4.34 ± 0.11	4.36 ± 0.08	4.44 ± 0.14	4.08 ± 0.64
F-VP		Vacuum	4.69 ± 0.34	4.21 ± 0.29	4.27 ± 0.01	4.59 ± 0.15	4.36 ± 0.03
C-SP	4 ºC	Standard	4.69 ± 0.34	4.29 ± 0.04	4.28 ± 0.03	4.42 ± 0.02	4.26 ± 0.04
C-VP		Vacuum	4.69 ± 0.34	4.23 ± 0.00	4.21 ± 0.08	4.41 ± 0.06	4.32 ± 0.08
RT-SP	22 ºC	Standard	4.69 ± 0.34	4.02 ± 0.08	4.13 ± 0.18	3.69 ± 0.24	3.62 ± 0.27
RT-VP		Vacuum	4.69 ± 0.34	4.10 ± 0.08	3.75 ± 0.41	3.34 ± 0.08	3.27 ± 0.05
HT-SP	37 ºC	Standard	4.69 ± 0.34	3.25 ± 0.30	2.91 ± 0.13	2.79 ± 0.01	2.60 ± 0.03
HT-VP		Vacuum	4.69 ± 0.34	3.13 ± 0.03	3.18 ± 0.31	2.91 ± 0.01	2.59 ± 0.09

Figure S4. Counts of Total Bacteria (log CFU g^{-1}) in FFP Samples Stored Under Different Storage Temperatures and Packaging Modes. FFP: fermented food product; F-SP: freezing standard packaging; F-VP: freezing vacuum packaging; C-SP: cooling standard packaging; C-VP: cooling vacuum packaging; RT-SP: room temperature standard packaging; RT-VP: room temperature vacuum packaging; HT-SP: high temperature standard packaging; HT-VP: high temperature vacuum packaging. Values are expressed as mean ± SD.



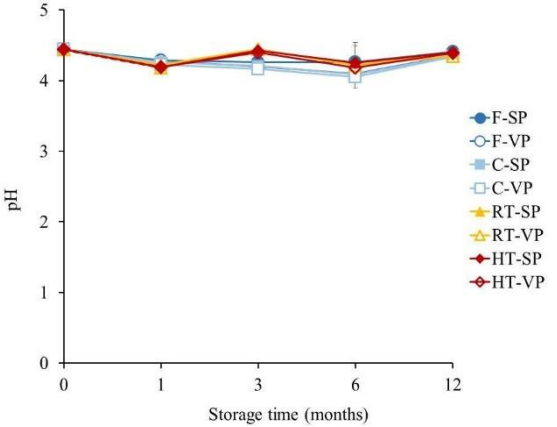
Sample Code	Temperature	Packaging	Storage Time (months)				
			0	1	3	6	12
F-SP	-20 ºC	Standard	7.44 ± 1.01	6.22 ± 0.06	6.70 ± 0.19	6.58 ± 0.17	6.72 ± 0.19
F-VP		Vacuum	7.44 ± 1.01	5.58 ± 0.28	6.82 ± 0.05	6.34 ± 0.03	6.30 ± 0.03
C-SP	4 ºC	Standard	7.44 ± 1.01	6.37 ± 0.01	5.83 ± 0.16	5.25 ± 0.10	4.22 ± 0.09
C-VP		Vacuum	7.44 ± 1.01	6.15 ± 0.00	5.89 ± 0.00	5.50 ± 0.42	4.70 ± 0.23
RT-SP	22 ºC	Standard	7.44 ± 1.01	4.35 ± 0.28	4.56 ± 0.09	3.57 ± 0.04	3.46 ± 0.01
RT-VP		Vacuum	7.44 ± 1.01	4.03 ± 0.35	3.91 ± 0.03	3.47 ± 0.08	3.42 ± 0.01
HT-SP	37 ºC	Standard	7.44 ± 1.01	3.32 ± 0.24	3.30 ± 0.03	3.82 ± 0.08	1.00 ± 0.00
HT-VP		Vacuum	7.44 ± 1.01	3.60 ± 0.04	3.25 ± 0.07	3.86 ± 0.49	1.00 ± 0.00

Figure S5. Counts of LAB (log CFU g^{-1}) in FFP Samples Stored Under Different Storage Temperatures and Packaging Modes. LAB: lactic acid bacteria; FFP: fermented food product; F-SP: freezing standard packaging; F-VP: freezing vacuum packaging; C-SP: cooling standard packaging; C- VP: cooling vacuum packaging; RT-SP: room temperature standard packaging; RT-VP: room temperature vacuum packaging; HT-SP: hightemperature standard packaging; HT-VP: high temperature vacuum packaging. Values are expressed as mean ± SD.



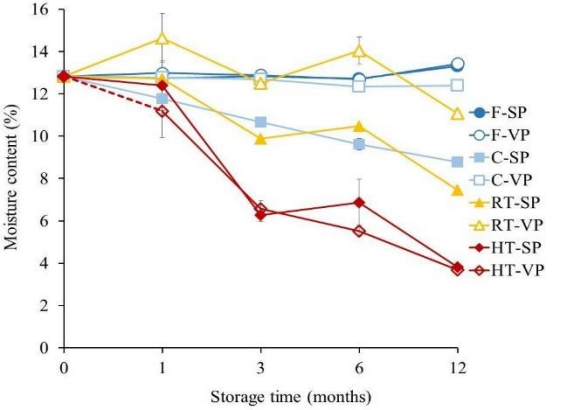
Sample Code	Temperature	Packaging	Storage Time (months)				
			0	1	3	6	12
F-SP	-20 ºC	Standard	3.16 ± 0.32	2.67 ± 0.11	3.25 ± 0.46	2.57 ± 0.04	2.34 ± 0.16
F-VP		Vacuum	3.16 ± 0.32	2.73 ± 0.22	2.74 ± 0.12	2.39 ± 0.09	2.65 ± 0.33
C-SP	4 ºC	Standard	3.16 ± 0.32	1.00 ± 0.00	1.43 ± 0.60	1.00 ± 0.00	1.00 ± 0.00
C-VP		Vacuum	3.16 ± 0.32	1.44 ± 0.62	2.90 ± 0.20	1.48 ± 0.67	1.00 ± 0.00
RT-SP	22 ºC	Standard	3.16 ± 0.32	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
RT-VP		Vacuum	3.16 ± 0.32	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
HT-SP	37 ºC	Standard	3.16 ± 0.32	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
HT-VP		Vacuum	3.16 ± 0.32	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00

Figure S6. Counts of yeasts (log CFU g^{-1}) in FFP Samples Stored Under Different Storage Temperatures and Packaging Modes. FFP: fermented food product; F-SP: freezing standard packaging; F-VP: freezing vacuum packaging; C-SP: cooling standard packaging; C-VP: cooling vacuum packaging; RT-SP: room temperature standard packaging; RT-VP: room temperature vacuum packaging; HT-SP: high temperature standard packaging; HT-VP: high temperature vacuum packaging. Values are expressed as mean ± SD.



Sample Code	Temperature	Packaging	Storage Time (months)				
			0	1	3	6	12
F-SP	-20 ºC	Standard	4.44 ± 0.00	4.29 ± 0.00	4.26 ± 0.01	4.26 ± 0.24	4.41 ± 0.01
F-VP		Vacuum	4.44 ± 0.00	4.28 ± 0.01	4.20 ± 0.02	4.10 ± 0.01	4.36 ± 0.01
C-SP	4 ºC	Standard	4.44 ± 0.00	4.26 ± 0.01	4.21 ± 0.01	4.09 ± 0.01	4.35 ± 0.02
C-VP		Vacuum	4.44 ± 0.00	4.23 ± 0.00	4.17 ± 0.03	4.05 ± 0.03	4.34 ± 0.00
RT-SP	22 ºC	Standard	4.44 ± 0.00	4.23 ± 0.01	4.44 ± 0.03	4.22 ± 0.32	4.40 ± 0.04
RT-VP		Vacuum	4.44 ± 0.00	4.18 ± 0.01	4.44 ± 0.01	4.22 ± 0.04	4.35 ± 0.06
HT-SP	37 ºC	Standard	4.44 ± 0.00	4.19 ± 0.04	4.43 ± 0.03	4.25 ± 0.02	4.40 ± 0.03
HT-VP		Vacuum	4.44 ± 0.00	4.19 ± 0.01	4.40 ± 0.01	4.18 ± 0.05	4.39 ± 0.02

Figure S7. pH in FFP Samples Stored Under Different Storage Temperatures and Packaging Modes. FFP: fermented food product; F-SP: freezing standard packaging; F-VP: freezing vacuum packaging; C-SP: cooling standard packaging; C-VP: cooling vacuum packaging; RT-SP: room temperature standard packaging; RT-VP: room temperature vacuum packaging; HT-SP: high temperature standard packaging; HT-VP: high temperature vacuum packaging. Values are expressed as mean ± SD.



Sample Code	Temperature	Packaging	Storage Time (months)				
			0	1	3	6	12
F-SP	-20 ºC	Standard	12.84 ± 0.21	12.75 ± 0.09	12.82 ± 0.13	12.73 ± 0.03	13.31 ± 0.13
F-VP		Vacuum	12.84 ± 0.21	12.98 ± 0.08	12.89 ± 0.12	12.69 ± 0.16	13.43 ± 0.16
C-SP	4 ºC	Standard	12.84 ± 0.21	11.78 ± 0.24	10.66 ± 0.24	9.62 ± 0.28	8.79 ± 0.06
C-VP		Vacuum	12.84 ± 0.21	12.76 ± 0.07	12.68 ± 0.01	12.35 ± 0.12	12.39 ± 0.22
RT-SP	22 ºC	Standard	12.84 ± 0.21	12.71 ± 0.11	9.88 ± 0.03	10.48 ± 0.06	7.45 ± 0.21
RT-VP		Vacuum	12.84 ± 0.21	14.64 ± 1.15	12.49 ± 0.01	14.05 ± 0.63	11.08 ± 0.21
HT-SP	37 ºC	Standard	12.84 ± 0.21	12.39 ± 1.17	6.28 ± 0.31	6.86 ± 1.12	3.83 ± 0.16
HT-VP		Vacuum	12.84 ± 0.21	11.19 ± 1.25	6.57 ± 0.37	5.51 ± 0.27	3.68 ± 0.06

Figure S8. Moisture Content (%) in FFP Samples Stored Under Different Storage Temperatures and Packaging Mode. FFP: fermented food product; F-SP: freezing standard packaging; F-VP: freezing vacuum packaging; C-SP: cooling standard packaging; C-VP: cooling vacuum packaging; RT-SP: room temperature standard packaging; RT-VP: room temperature vacuum packaging; HT-SP: high temperature standard packaging; HT-VP: high temperature vacuum packaging. Values are expressed as mean ± SD.

Table S1. Report of Model Coefficient Values, Confidence Intervals and *p*-values of the Total Bacteria Generalized Linear Model Analysis (GLM).

Parameter	Storage time (months)	Condition	β	95% CI		*p*-value
Temperature	1	F	0.315	-0.049	0.679	0.081
		C	0.270	-0.094	0.634	0.126
		HT	-0.770	-1.134	-0.406	0.001
	3	F	0.225	-0.242	0.692	0.299
		C	0.150	-0.317	0.617	0.480
		HT	-1.220	-1.687	-0.753	0.000
	6	F	0.750	0.477	1.023	0.000
		C	0.725	0.452	0.998	0.000
		HT	-0.900	-1.173	-0.627	0.000
	12	F	0.455	-0.126	1.036	0.180
		C	0.635	0.054	1.216	0.036
		HT	-1.020	-1.601	-0.439	0.004
Packaging	1	VP	0.075	-0.289	0.439	0.647
	3		-0.380	-0.847	0.087	0.097
	6		-0.350	-0.623	-0.077	0.018
	12		-0.355	-0.936	0.226	0.196

Comparisons were computed between the different conditions of temperature and packaging and its reference condition, RT and SP respectively. CI: confidence interval; F: freezing; C: cooling; HT: high temperature; VP: vacuum packaging; RT: room temperature; SP: standard packaging.

Table S2. Report of Model Coefficient Values, Confidence Intervals and p-values of LAB Generalized Linear Model Analysis (GLM).

Parameter	Storage time (months)	Condition	β	95% CI		*p*-value
Temperature	1	F	1.875	1.397	2.353	0.000
		C	2.025	1.547	2.503	0.000
		HT	-1.025	-1.503	-0.547	0.001
	3	F	2.140	1.912	2.368	0.000
		C	1.275	1.047	1.503	0.000
		HT	-1.255	-1.483	-1.027	0.000
	6	F	3.015	2.458	3.572	0.000
		C	1.685	1.128	2.242	0.000
		HT	0.255	-0.302	0.812	0.322
	12	F	3.260	3.002	3.518	0.000
		C	0.760	0.502	1.018	0.000
		HT	-2.455	-2.713	-2.197	0.000
Packaging	1	VP	-0.315	-0.793	0.163	0.167
	3		-0.645	-0.873	-0.417	0.000
	6		-0.095	-0.652	0.462	0.704
	12		-0.035	-0.293	0.223	0.763

Comparisons were computed between the different conditions of temperature and packaging and its reference condition, RT and SP respectively. LAB: lactic acid bacteria; CI: confidence interval; F: freezing; C: cooling; HT: high temperature; VP: vacuum packaging; RT: room temperature; SP: standard packaging.

Table S3. Report of Model Coefficient Values, Confidence Intervals and *p*-values of the Yeasts Generalized Linear Model Analysis (GLM).

Parameter	Storage time (months)	Condition	β	95% CI		*p*-value
Temperature	1	F	1.670	1.255	2.085	0.000
		C	0.415	0.000	0.830	0.050
		HT	-1.11e-16	-0.415	0.415	1.000
	3	F	2.245	1.671	2.819	0.000
		C	0.725	0.151	1.299	0.019
		HT	-7.307e-17	-0.574	0.574	1.000
	6	F	1.590	1.394	1.786	0.000
		C	0.930	0.734	1.126	0.000
		HT	8.568e-16	-0.196	0.196	1.000
	12	F	1.340	1.041	1.639	0.000
		C	-4.935e-16	-0.299	0.299	1.000
		HT	-4.935e-16	-0.299	0.299	1.000
Packaging	1	VP	0.200	-0.215	0.615	0.298
	3		-1.388e-17	-0.574	0.574	1.000
	6		8.327e-16	-0.196	0.196	1.000
	12		-8.755e-16	-0.299	0.299	1.000

Comparisons were computed between the different conditions of temperature and packaging and its reference condition, RT and SP respectively. CI: confidence interval; F: freezing; C: cooling; HT: high temperature; VP: vacuum packaging; RT: room temperature; SP: standard packaging.

Table S4. Report of Model Coefficient Values, Confidence Intervals and *p*-values of the pH Generalized Linear Model Analysis (GLM).

Parameter	Storage time (months)	Condition	β	95% CI		*p*-value
Temperature	1	F	0.060	0.019	0.101	0.010
		C	0.035	-0.006	0.076	0.086
		HT	-0.035	-0.076	0.006	0.086
	3	F	-0.185	-0.230	-0.140	0.000
		C	-0.235	-0.280	-0.190	0.000
		HT	-0.010	-0.055	0.035	0.622
	6	F	0.045	-0.285	0.375	0.762
		C	-0.125	-0.455	0.205	0.408
		HT	0.030	-0.300	0.360	0.839
	12	F	0.010	-0.058	0.078	0.743
		C	-0.055	-0.123	0.013	0.099
		HT	-5.329e-15	-0.068	0.068	1.000
Packaging	1	VP	-0.045	-0.086	-0.004	0.036
	3		-0.005	-0.050	0.040	0.804
	6		0.000	-0.330	0.330	1.000
	12		-0.050	-0.118	0.018	0.128

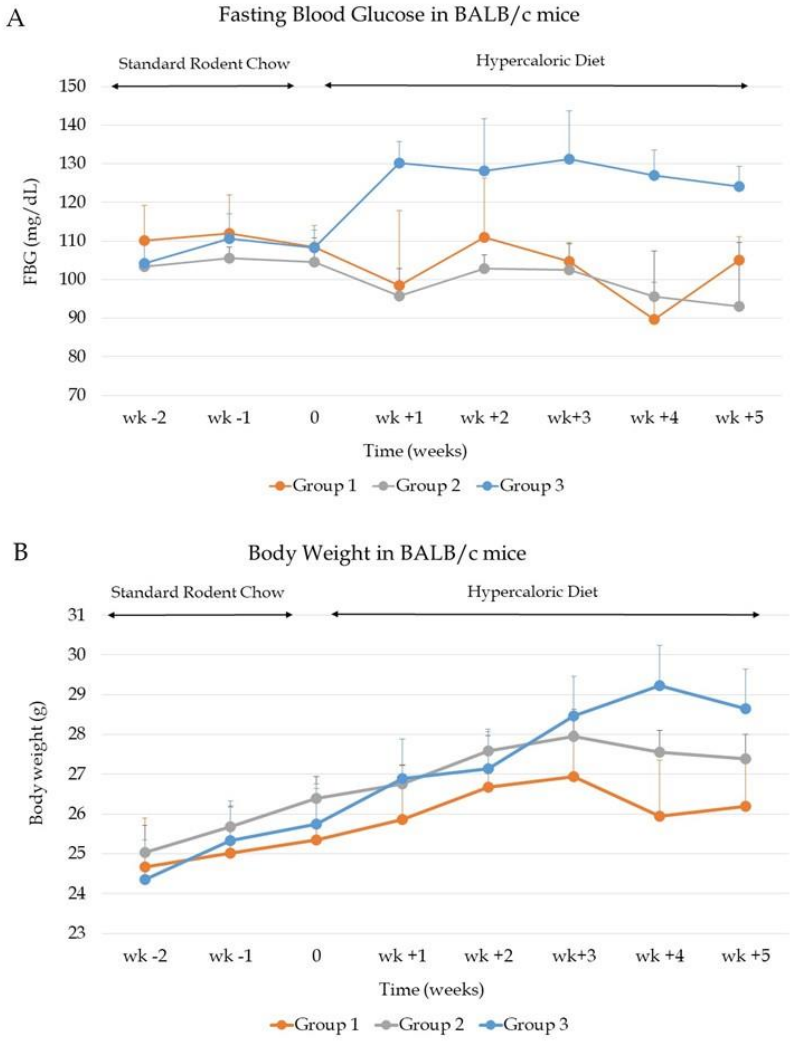
Comparisons were computed between the different conditions of temperature and packaging and its reference condition, RT and SP respectively. CI: confidence interval; F: freezing; C: cooling; HT: high temperature; VP: vacuum packaging; RT: room temperature; SP: standard packaging.

Table S5. Report of Model Coefficient Values, Confidence Intervals and *p*-values of the Moisture Content (%) Generalized Linear Model Analysis (GLM).

Parameter	Storage time (months)	Condition	β	95% CI		*p*-value
Temperature	1	F	0.035	-1.939	2.009	0.968
		C	-0.930	-2.904	1.044	0.309
		HT	-0.320	-2.294	1.654	0.718
	3	F	2.940	2.474	3.406	0.000
		C	0.780	0.314	1.246	0.005
		HT	-3.600	-4.066	-3.134	0.000
	6	F	1.255	0.144	2.366	0.031
		C	-1.855	-2.966	-0.744	0.005
		HT	-4.620	-5.731	-3.509	0.000
	12	F	5.865	5.495	6.235	0.000
		C	1.345	0.975	1.715	0.000
		HT	-3.620	-3.990	-3.250	0.000
Packaging	1	VP	1.925	-0.049	3.899	0.055
	3		2.605	2.139	3.071	0.000
	6		2.570	1.459	3.681	0.001
	12		3.630	3.260	4.000	0.000

Comparisons were computed between the different conditions of temperature and packaging and its reference condition, RT and SP respectively. CI: confidence interval; F: freezing; C: cooling; HT: high temperature; VP: vacuum packaging; RT: room temperature; SP: standard packaging.

Supplementary Figures General Discussion



Supplementary Figure 1. Representation of FBG (A) body weight (B) in BALB/c mice supplemented with different formats of Probisan® that differ in the microbial fraction. Group 1: Mice fed stored Probisan® *ad libitum* during 5 weeks; Group 2: Mice fed freshly produced Probisan® *ad libitum* during 5 weeks; Group 3: Mice fed Probisan® elaborated without the incorporation of the fermentative inoculum.

Supplementary Figure 2. Representative images of bread loaves and bread slices prepared with different Probisan® quantities (0, 5, 10 and 15%).