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# *In vitro* simulation of gastrointestinal tract toward evaluation of performance of probiotic strains – a systematic review

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"You should enjoy the little detours to the fullest. Because that's where you'll find things more importante than what you want"

Ging Freecss

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## ABSTRACT

The ability to endure the conditions found along the human gastrointestinal (GI) tract and still remain viable is crucial for a microorganism exert positive effects in the host's health and, therefore, to be regarded as potential probiotic. In the past few years, the increasing number of lactose-intolerant individuals led to a quest for new probiotic carriers and, consequently, probiotic sources – with table olives appearing as a promising candidate. Our review of *in vitro* methods for assessment of resistance of isolates from table olives to GI tract conditions unfolded significant variations between the protocols reported. Among the studies analyzed, 10 out of a total of 23 performed independent tests to assess resistance to the environment prevailing in the different organs of the GI tract (stomach and intestine), while the remaining methodologies covered a sequential simulation of GI tract – where the suspensions were exposed to sucessive stresses, as happens in the actual GI tract, either via static or dynamic processes. Existence of an oral phase of digestion was considered as such in only two studies. The main stresses evaluated were the highly acidic pH experience in the stomach and the antimicrobial action of the bile segregated in the intestine; the presence of dominant enzymes of the corresponding organs was also considered in several reports. A critical assessment and comparison between the methods employed contributed to a better understanding of the best ways to simulate *in vivo* GI tract resistance, in attempts to produce a better representation of reality. In all reports, the isolates from table olives were either lactic acid bacteria or yeasts, or both. The ultimate objective of this master thesis was to recommend a protocol suitable for evaluation of the ability/resistance of native lactic acid bacteria and yeasts to passage through the GI tract.

**Keywords:** table olives, lactic acid bacteria, yeasts, digestion, resistance

# RESUMO

A capacidade de suportar as condições encontradas ao longo do trato GI humano (GI) e ainda permanecer viável é crucial para que um microorganismo exerça efeitos positivos na saúde do hospedeiro e, portanto, seja considerado um potencial probiótico. Nos últimos anos, o número crescente de indivíduos intolerantes à lactose levou a uma procura por novos transportadores de probióticos e, consequentemente, fontes de probióticos - com azeitonas de mesa a surgir como um candidato promissor. A revisão de métodos *in vitro* para avaliação da resistência de isolados de azeitonas de mesa a condições do trato GI revelou variações significativas entre os protocolos reportados. Entre os estudos analisados, 10 de um total de 23 realizaram testes independentes para avaliar a resistência ao meio ambiente prevalecente nos diferentes órgãos do trato GI (estômago e intestino), enquanto as restantes metodologias recorreram uma simulação sequencial do trato GI - onde os as suspensões foram expostas a stresses sucessivos, como acontece no trato GI real, por processos estáticos ou dinâmicos. A existência de uma fase oral da digestão foi considerada em apenas dois estudos. Os principais obstáculos à sobrevivência das estirpes avaliados foram a experiência de pH altamente ácido no estômago e a ação antimicrobiana da bile segregada no intestino; a presença das principais enzimas segregadas nos órgãos correspondentes também foi considerada em vários estudos. Uma avaliação crítica e comparação entre os métodos utilizados contribuiu para uma melhor compreensão das melhores metodologias para simular a resistência do trato GI *in vivo*, na tentativa de produzir uma melhor representação da realidade. Em todos os estudos, os isolados das azeitonas de mesa eram bactérias do ácido láctico ou leveduras, ou ambas. O objetivo final desta dissertação de mestrado foi recomendar um protocolo apropriado para avaliação da capacidade / resistência de bactérias do ácido láctico e leveduras nativas à passagem pelo trato GI.
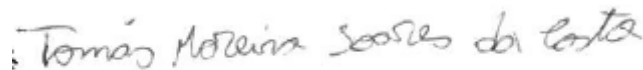
**Palavras-chave:** azeitonas de mesa, bactérias do ácido lático, leveduras, digestão, resistência

## DECLARATION

I now declare that this thesis is my own work and that, to the best of my knowledge and belief, it contains no material previously published or produced by another party in fulfilment, partial or otherwise, of any other degree or diploma at another University or Institute of higher learning except where due acknowledgment is made in the text.

Declaro que esta tese é um trabalho meu e que, segundo o meu conhecimento e crença, não contém material publicado anteriormente ou produzido por outra parte em cumprimento, parcial ou não, de qualquer outro grau ou diploma de outra Universidade ou Instituto de ensino superior, exceto quando o devido reconhecimento for feito no texto.

June 29th 2020 _____

(Tomás Moreira Soares da Costa)

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# 1. Introduction

## 1.1.　Objectives

Indigenous microbiota from fermented vegetable aliments have been emerging as a promising probiotic due to their structure and composition. In the last few years, studies pertaining the ability of these microorganisms to resist the harsh environment of the human gut have been increasing. Table olives are seen as an interesting food carrier for probiotics; and many studies have recently investigated probiotic traits from microbiota isolated from this vegetable. However, there is no consensus on the methodology to assess the GI resistance of microorganisms adventitious in olives, since different approaches have been followed. Therefore, this master thesis consisted of a review of methodologies employed in the past ten years to study resistance to the passage through the gastrointestinal (GI) tract of microorganisms isolated from table olives – one of the main features to be claimed as probiotic. Additionally, it entailed a critical assessment of the methodologies reported, with discussion of their strengths and weaknesses.

## 1.2.　Relevance of Work

Probiotics for ingestion have been used mostly in the dairy industry for several decades now. However, the number of lactose-intolerant individuals have been increasing in recent years. Hence, several types of food have been investigated as possible probiotic carriers without causing harm to the consumers, like allergies or intolerances – with a particular emphasis on fermented vegetable foods, as is the case of table olives. The process of production of these fermented products frequently involves harsh conditions like extreme pH values, salt concentration and presence of antimicrobial compounds, which prove a hardship to most already known probiotics from dairy origin. Therefore, it is essential to evaluate potential probiotic features in the indigenous microbiota of these products.

Since microorganisms must survive the GI tract environment to eventually exert their beneficial properties in the colon, the methodology applied to study their survival should be the most efficient and close to reality as possible. The literature encompasses distinct methods, resorting to different kind of tests, thereby making it hard to decide which method to choose. Therefore, a compilation and review of methods appears useful toward a preliminary, yet broad and critical analysis that

supports an educated choice of protocol for studying resistance to digestion that produces reliable results.

## 1.3.      Thesis Outline

This dissertation is divided into 6 sections. The first section, denominated "Introduction", covers the general framework and presentation of the project, and its objectives, and description of the dissertation organization. Then, the second section, "Contextualization", provides the general background on the theme, from the production of table olives, to the identification and selection of probiotic strains. In the third section, the method of search and selection of the methods to be included in the review were described. The fourth section, "Results and Discussion" comprises the results of the review, with a comparison between the methods and a critical assessment of the methods employed. The fifth section concerns to the conclusions of the project, and their subsequent effect upon future work; while the sixth section pertains to the constraints imposed upon the dissertation. Lastly, a "References" section is provided.

## 2. Contextualization

### 2.1.    Table Olives

Table olives are the fermented fruit of the olive tree (*Olea europaea L.*), a small tree belonging to the family *Oleaceae*, native from tropical and warm temperate regions of the world. Olive trees are traditionally cultivated in the coastal areas of the eastern Mediterranean Basin, the adjoining coastal areas of southeastern Europe, western Asia and northern Africa [1]. Its fruit, also called olive, has extreme commercial prominence in the Mediterranean region as prime source of olive oil - its nuclear dietary fat.

Table olives and olive oil are two core ingredients of the Mediterranean diet. This diet has potential beneficial effects upon human health due to the phenolic composition that supports several functional properties, such as antioxidant, anticarcinogenic, anti-inflammatory, antimicrobial, antihypertensive, antilipidemic, cardiotonic, laxative and antiplatelet [1].

Olive is a rich source of valuable nutrients and present bioactivities of medicinal and therapeutic interest, with an average composition of water (50%), protein (1.6%), oil (22%), carbohydrate (19.1%), cellulose (5.8%), inorganic substances (1.5%) and phenolic compounds (1–3%) [1]. Phenolic compounds are responsible for the browning in the olive fruits and change qualitatively and quantitatively during fruit maturation. The olive fruit is constituted by 3 parts, epicarp or skin, mesocarp or pulp and endocarp or stone. The epicarp is covered with wax; during the growth phase, the skin colour turns from light green to purple and brown or black. Although the average growth and ripening of the fruit takes place for ca. 5 months in the usual climatic conditions prevailing during cultivation, it can be slower in colder weather [2]. Olive phenols contribute towards the sensory and aromatic characteristics of the fruit.

Oleuropein is the main phenolic compound present in olives before processing, and the bitterness of the fruit is mainly attributed to the presence of this compound. Hence, olive fruits are rarely used in their natural form, being typically subjected to fermentation or cured with lye or brine to degrade the bitter components and make them more palatable [3].

Production of table olives usually follows one of three internationally recognized practices: Spanish-style green olives, Californian-style black olives and Greek-style natural black olives. In the Spanish-style method, the bitterness of the olive is initially removed, and the olive pericarp is permeabilized via addition of sodium hydroxide as food-grade treatment; they are then rinsed and fermented, mainly via lactic

fermentation, in brine for several months [4]. Alternatively, Greek-style olives are placed directly in the brine to remove oleuropein partially or in full, undergo a mixed-acid fermentation, chiefly by the action of yeasts. Conversely, Californian-style black olives are treated directly with lye and oxidized, then washed, placed in brine, and packed in cans with heat-sterilization – so they do not undergo fermentation at all [1]. However, this chemical treatment, which assures the chemical degradation of oleuropein, also leads to high losses in the nutrient profile of the processed olives [5]. The different techniques for the preparation of table olives are presented in Figure 1.

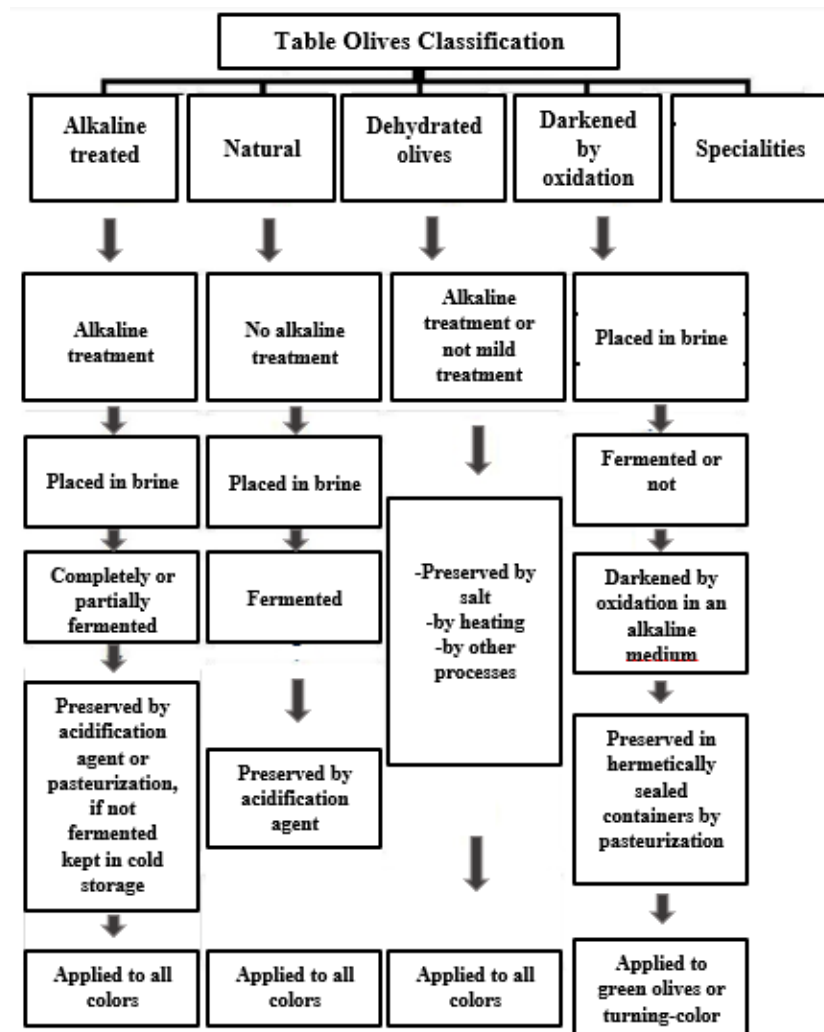**Figure 1.** Classification of table olives according to preparation method. Adapted from Heperkan et al. [6]

Fermented olives are considered as a probiotic food, since some of the microorganisms present can survive transit through the GI tract – and they can exert antimicrobial effects against local pathogens in the intestinal epithelial cells, among other health effects [3]. Moreover, table olives could be regarded as a promising

probiotic food – considering that, compared to dairy products, they do not pose problems to those people who are intolerant to milk and milk products, or those who need low cholesterol diets [7, 8]. Hence, awareness of its probiotic potential is expected to increase significantly the (already notable) nutritional value of table olives [9].

### 2.1.1. Table Olive Fermentation

Fermentation is a process dependent on the biological activity of microorganisms for the production of a range of metabolites and is the oldest biotechnological method to preserve vegetables [9]; it has indeed been used by Mankind as a way to store food since ancient times. Microorganisms exert catabolic activity to breakdown complex compounds, thus releasing free fatty acids, amino acids, and simple sugars while synthesizing several vitamins and bioactive compounds. Therefore, fermentation can affect the nutritional quality of food, as it can induce physicochemical changes that improve nutrient density and increase their bioavailability. In the particular case of fermented olives, lactic acid fermentation takes a specific relevance upon the conversion of carbohydrates to organic acids – usually catalysed by yeasts, bacteria, moulds, or a combination of more than one single organism [3]. The microbiota responsible for olive fermentation often break down carbohydrates, proteins, and lipids present in the raw materials by secreting enzymes, enhanced by the acidic nature of fermentation – which constitutes a nutritionally desirable event, as it turns the food more easily digestible. Moreover, fermentation reduces the levels of certain antinutritional factors that interfere with digestion, thus making nutrients more efficiently utilized along the human digestive tract, and the final product safer for consumption at large [3, 10].

In natural olive fermentations the final product is obtained in brine by interactions between microorganisms, and their metabolism is essential for the transformation of natural substrates towards the improvement of nutritional value, appearance and flavour, while favouring degradation of undesirable factors and contributing to make safer products. Table olive fermentation often occurs spontaneously by action of the endogenous microbiota of the olive, without adding any starter culture, as soon as olives are put in brines [6]. Spontaneous fermentation occurs for 8 to 12 months, and is mainly driven by microbial populations of yeasts and LAB [11]. Bleve et al. [11] found that for the Italian Cellina di Nardò and Leccino table olives, the first part of the fermentation is accounted for by yeasts; and then LAB together with yeasts complete the process, for the period ranging from 90th day to 180th day. Spontaneous

fermentation processes are hardly predictable, due to a strong influence from the autochthonous microbiota [12].

Diverse microbial populations are involved in olive fermentation, mainly lactic acid bacteria (LAB), with particular relevance for *Lactobacillus plantarum* and *Lactobacillus pentosus*, and yeasts [13]. However, *Enterobacteriaceae, Clostridium, Pseudomonas*, and *Staphylococcus* and occasionally molds may also be present; despite their presence at the beginning of fermentation, the majority of this microorganisms are unable to survive until its end, due to the low pH [11]. Although initial conditions have to be considered, it is established that LAB grow spontaneously in treated olives, but yeasts can substitute them in natural olives. As previously noted, lactic acid fermentation is the predominant biological preservation method that contributes to a unique flavour development, and is considered to be the major contributor to the beneficial characteristics observed in table olives [6].

The ability of LAB to convert fermentable sugars to lactic acid and other organic acids, depending on their metabolic pathways, makes them the most important group of bacteria in olives [6]. Their action in spontaneous fermentation processes constitutes a fundamental step, due to a reduction of pH that leads to an acid degradation of oleuropein, while providing microbial stability in that it avoids growth of spoilage and pathogenic microorganisms. Furthermore, it promotes the development of a correct flavour and texture profile in the final product, enhances the fermentation process, and extends the shelf-life of the product [3]. On the other hand, yeasts have an essential contribution in the table olive production process, mainly by producing desirable metabolites and volatile compounds, such as ethanol, glycerol, higher alcohols, and esters – able to improve the organoleptic characteristics of the final product [4, 12, 14]. In addition, yeasts are capable of synthesizing vitamins, amino acids, and purines, and also hydrolyse complex carbohydrates essential to enhance LAB growth, as well as exhibit activity against undesired microbiota and aid in degradation of phenolic compounds like oleuropein (that often inhibit LAB development) [11, 13]. The hydrolysis of the raw materials causes changes to the environment, sometimes undergoing a pH drop. Moreover, peptides and amino acids formed can be converted to odoriferous organoleptic compounds, thus improving the flavour of the fermented olives. However, some issues are associated with the presence of yeasts during the fermentation process, such as formation of gas pockets, softening of the olive tissue, package bulging, clouding of brines, and production of off-flavours [6, 13].

Besides the indigenous microbiota, the fermentation processes are greatly influenced by methodological factors, such as fermentation temperature and salt concentration in the brines. The physico-chemical conditions, the availability of fermentable substrates and the salt content may as a whole lead to products with different characteristics and quality, or even problems with spoilage leading to abnormal fermentation and a defective final product. To get a better control of fermentation process, and reduce the risk of growth of undesirable strains, the application of starter cultures has been a rising procedure, recently [12]. Starter cultures not only decrease the risk of spoilage, but also accelerate acidification of the brine and make it more effective, and reduce the metabolic energy required during the process [6, 10]. The possible use of a probiotic strain as a starter to drive the fermentation would be highly advisable, since the use of a different starter strain could hamper the probiotic's multiplication and survival by depletion of nutrients in brine [16].

### 2.1.2. Table Olives as Source and Food Matrix/Carriers for Probiotics

Nowadays, the number of lactose-intolerant, vegetarian, allergic, and dyslipidemic individuals have justified a growing demand for non-dairy probiotic products. In this regard, probiotic vegetable preparations could attract more consumers, and vegetable products as new carrier matrices of probiotics are currently of increasing interest. Physico-chemical properties of food carriers used for probiotic delivery, such as buffering capacity and pH, are significant factors that influence the survival of the probiotic strain, and thus dispay of probiotic effects during gastric transit [8, 9]. Table olives represent an excellent vehicle to transport probiotics both for their microarchitecture and for the presence of nutrients. The skin of olives contains microscopic pores, called stomata, used as primary portals for entrance and exit of solutes and gases. However, the location of the pores at the interface between internal plant tissues and the environment makes them convenient gates for endophytic bacteria colonization. Studies proved that this microstructure of the olive works as a protection to the probiotics, by ensuring cell integrity as they cross the GI tract. However, only a few probiotic cultures used with success in dairy products exhibit acceptable viabilities in plant matrices by the time of consumption, due to the harsh environmental conditions prevailing in plant matrices - e.g. high osmotic pressure, poor nutrient profile, and presence of antibacterial compounds [6, 8]. Therefore, screening for LAB strains of plant origin for potential probiotic features may help overcome such technological challenges [9]. Moreover, probiotics of vegetable

origin exhibit unique survival characteristics due to the natural presence of high amounts of prebiotics in the plant material, which improve their functional efficacy with increased resistance to the acidic environment during gastric transit [11, 12]. Furthermore, those food matrices are characterized by intrinsic physico-chemical properties that mimic conditions in the GI tract. Probiotic bacteria from vegetables or fruits possess mechanisms for adherence to surfaces similar to those prevailing on the intestinal surface, along with their tolerance to acids and several other stresses [18]. LAB have been shown to enter through stomata and predominate in the intercellular space of the substomal cells. This ability of LAB to colonize the surface of olives and form biofilms during fermentation, in combination with the microstructure of the olive surface that protects microorganisms during digestion, unfold new perspectives for the use of table olives as a wild source of LAB with probiotic and technological features [12]. Furthermore, the ingestion of a healthy dose of table olives (10-15 olives) can carry $10^9$-$10^{10}$ CFU of selected *Lactobacillus* spp., thus allowing the ingestion of cell numbers comparable to milk-based probiotic products [9].

## 2.2.    Selection of Probiotic Strains

According to the Food and Agriculture Organization of the United Nations and the World Health Organization (FAO/WHO) [19], probiotics are defined as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host". Probiotic foods are, in general, fermented foods containing live microorganisms in an adequate amount to reach the intestine. They contribute to the intestinal microflora balance of the host, by stimulation of the beneficial microorganisms and reduction of pathogens [20]. Probiotics increase intestinal mucin production, which prevent the attachment of enteropathogens by steric hindrance, or through competitive inhibition for mucin attachment sites. Probiotics also increase the production of antimicrobial peptides, and decrease the epithelial permeability to intraluminal pathogens and toxins. They may even reduce the exposure to chemical carcinogens, thus playing a detoxifying role. These probiotics exhibit a large number of health effects. They are currently used in the prevention or treatment of several diseases, such as rotavirus-induced diarrhea, antibiotic-associated diarrhea, allergies and atopic diseases in children, and the modulation of human gut microbiota and immunomodulation [9, 20, 21].

Most physiological properties studies were performed with human and animal internal cavities strains, essentially *Lactobacillus* and *Bifidobacterium* – considering that these strains would be better adapted to colonize the human/animal GI tract [22,

23]. On the other hand, research has started to grow on probiotic functions of LAB from foods like dairy products, dry sausages, foods of plant origin, fruits, cereals, meat, or fish [25]. Probiotic foods have accordingly been restricted almost exclusively to dairy products, and have encompassed mostly *Bifidobacterium* spp [3]. However, vegetable matrices, such as fermented olives, constitute a rich alternative source of probiotic microorganisms, mainly LAB and yeasts [3, 12]. Each new proposed probiotic strain must be characterized *per se*, since it is widely recognized that probiotic features are strictly strain-specific and cannot be considered as typical characteristics of a bacterial species at large. Hence, for a microorganism strain to be regarded as a probiotic, it should exhibit particular characteristics and several guidelines must be followed to ascertain them.

### 2.2.1. Resistance to the GI tract environment

In order to reach the intestine while alive, microorganisms pass along the human GI tract, since they are ingested in the mouth until they the large intestine. Therefore, it is essential that they have protection systems to withstand the wide range of pH experienced along digestion, apart from high concentrations of many digestive enzymes and other substances segregated, as bile salts [27, 28]. The stomach is known to have a very acidic environment, and there is segregation of several enzymes, like pepsin and lipases. Furthermore, in the intestine, bile acids exhibit detergent actions that are unfavorable to many microorganisms, which make it difficult their survival and growth in the intestinal tract [28]. Hence, in the first place, microorganisms with probiotic potential must survive gastric juices and be able to grow in the presence of bile, to reach the intestine in an adequate amount while alive, so they should be resistant to the simulated conditions prevailing during gastric and pancreatic digestion [12, 30]. Resistance to GI conditions *in vitro* is somehow useful, despite its limited predictability due to the inaccessibility of sophisticated and dynamic, computer-controlled models of the GI tract to most laboratories [31–33]. These *in vitro* assessments of digestion resistance differ by the transit time, the form of probiotic assimilation (alone or inserted in food matrices), and the complexity of the GI model [7].

Although *in vitro* assays may provide information on acid tolerance, the method still needs refinement to correlate with *in vivo* tests, or validation by human clinical trials [23]. *In vivo* tests are very important also because strain survival partially depends, as previously described, on the potential protective action of the associated carrier food which relates in some way to its chemical composition [33].

### 2.2.2.Gut adhesion and colonization

Another critical feature is the ability of the selected strain to colonise the digestive tract and, therefore, to adhere to the intestinal mucosa and enterocytes; this must happen in order to allow strains to remain in the intestinal tract and exert their potential health benefits in the host, such as excluding pathogenic bacteria through competition for binding sites [29, 30]. The human intestine is colonized by a large number of microorganisms that inhabit the intestinal tract and support a variety of physiological functions. This colonization begins at the birth of the individual. It is highly influenced by the environment where the early stages of life are spent, until a more stable intestinal microbiota forms that is different for each subject and plays essential functions toward host health [23, 31]. On the one hand, it brings metabolic benefits, playing a significant nutritional role, facilitating rapid salvage of energy from many nutrients, and providing diverse metabolic functions; this enables the host to survive in different nutritional environments, without having to adapt or develop all digestive processes [37]. On the other hand, symbiotic bacteria confer the host with several functions that promote immune homeostasis, immune responses and protection against pathogen colonization. Microbiota in the intestinal tract regulates the systemic and local immune responsiveness by affecting the development of gut-associated lymphoid tissue at an early age [23].

The epithelial cells of the intestine are covered by a protective layer of mucus, which offers attachment sites for gut bacteria [27]. This attachment of probiotics to the epithelial cells of the intestine is only temporary, and may bring several immunomodulatory benefits. The adhesion of microorganisms, like bacteria or yeasts, to animal cells involves much more complex processes than adhesion to synthetic surfaces, due mainly to the complexity of both biologic surfaces, and the ability of living cells to regulate the expression of molecules on their surface in response to changes in the environment [35, 38]. The adhesive properties of lactobacilli are directly linked to their surface properties, which are influenced by the structure and composition of their cell wall. Although the mechanisms of adhesion are not fully understood pertaining to bacterial cell-surface-associated proteins with mucus- and intestinal cells, binding properties have been identified and characterized in probiotic strains [25, 37]. Furthermore, the adhesion process appears to be related to the junction of several features of the bacterial or yeast cell. Cell surface hydrophobicity is a desired property of probiotics pertaining to their ability to adhere to epithelial cells, since it is suggested that bacteria with higher hydrophobicity undergo stronger adherence to intestinal cells Aggregation is also of significant importance for probiotic

strains, once it relates to their ability to adhere to epithelial cells. Auto-aggregation is essential toward adhesion to host cells as multiple aggregates, and the subsequent displacement of pathogens. At the same time, co-aggregation may even play an important role in the inhibition of the colonization by pathogenic bacteria, such as *Escherichia coli*, *Salmonella*, *Listeria innocua*, and *Staphilococcus aureus* [18, 34]. For example, LAB show beneficial impact of colonization inside GI tract through auto-aggregation, thus making this property of significant importance for probiotic strains – as they rely on this ability to adhere onto epithelial cells [40]. As enteropathogenic, *Escherichia coli* binds to epithelial cells via mannose receptors, thus allowing enterotoxin release. Probiotic strains with similar adherence capabilities could inhibit pathogen attachment and colonization at these binding sites, and thereby protect the host against infection [23, 35]. *In vitro* studies with human colorectal adenocarcinoma cell lines (Caco-2 or HT-29 cells), immobilised intestinal mucus and extracellular matrices have been extensively used as a system for assessing the adhesion capacity of lactobacilli, as well as their probiotic effects, such as protection against intestinal injury induced by pathogens [41].

The action of probiotics on the intestine tract is not limited to influence of intestinal flora; it also affects other organs, by modulating the immunological parameters and intestinal permeability, and by producing bioactive or regulatory metabolites [42]. Depending on the probiotic strain employed, immune responses can be either stimulated or suppressed, and many different immunological aspects may be affected. Stimulation of immunity can occur by increasing mucosal antibody production, boosting pro-inflammatory cytokine expression, and enhancing host defense, while suppressive effects are made apparent through decreasing cytokine expression, systemic inflammation, cellular proliferation, and increasing apoptosis [43]. Several known probiotics were shown to differentially induce *in vitro* maturation and cytokine expression of murine dendritic cells, with the possibility to favor T helper 1 (Th1), Th2 or Th3 immune responses [24]. Furthermore, exopolysaccharides (EPS) produced by LAB can induce immunological responses. The EPS produced by some food-grade genera, including *Bifidobacterium*, *Lactococcus* and *Lactobacillus*, have indeed been reported to be immunostimulatory [44].

### 2.2.3. Antimicrobial Activity

Besides GI resistance and ability to colonise the digestive tract, antimicrobial activity against pathogens is also a selection criterium. Different probiotics produce differing levels of bactericidal proteins, with various degrees of efficacy against

enteric pathogens [45]. LAB are known to produce antimicrobial substances, mainly organic acids and bacteriocins. The bacteriocins from LAB are bioactive peptides, derived from ribosomal-synthesized precursors, and with a bactericidal effect on a number of different gram-positive bacteria [46]. Organic acids act by creating an acidic environment that is inhibitory to pathogens. Additionally, studies confirmed that certain LAB strains are able to produce proteinaceous antifungal substances that exhibit inhibitory activity against a broad range of filamentous fungi and spoilage yeasts [46]. Particularly, the probiotic, *Lactobacillus reuteri*, produces an antimicrobial agent, reuterin, which has broad-spectrum activity against a variety of pathogens including bacteria, fungi, protozoa and viruses [43]. Moreover, antimicrobial activity can be effected through competitive action on nutrients and inhibition of binding due to competition, related to probiotic ability to colonize the gut, as previously described.

### 2.2.4. Bile Salt Hydrolase Activity

Bile salt hydrolase (BSH) activity is also considered to be an important property for probiotic strains. Microbial BSH function in bile salts detoxification may contribute to intestinal survival and persistence of producing strains, and boost their survival in the hostile conditions of the GI tract. Additionally, the amino acids released from bile salt detoxification may be used as carbon, nitrogen, and energy sources, thus granting a nutritional advantage to hydrolytic strains [12]. Cholesterol and bile salt metabolism are closely linked, where cholesterol is the precursor for synthesis of bile salts as water-soluble excretory end-product [23]. Hence, BSH activity facilitates incorporation of cholesterol or bile into bacterial membranes, and thus increases the tensile strength of the membranes [12]. However, an excessive deconjugation of bile salts could generate dysbiosis.

### 2.2.5. Safety

The safety of a strain to be used as a probiotic for human consumption is another important feature to bear in mind. Unless the strain with probiotic potential is safe for consumption, it cannot be used in the production of fermented foods. Several traits of the strain must be ensured before it is regarded as safe, and consequently considered as a probiotic. First, analysis of virulence determinants such as transmissible antibiotic resistance is imperative; strains harbouring resistance genes can in fact become a source of more harmful microorganisms (pathogens) that could be present in the gut

and other body sites [31, 41]. Furthermore, some microorganisms are able to produce biogenic amines, such as histamine and tyramine, through the activity of amino acid decarboxylases [47, 48]. It is also crucial to assess the production of these substances, since their occurrence in food has been associated with toxicological effects. Lack of mucin degradation has been reported as an essential marker for safety assessment of potential probiotic strains, since these glycosylated proteins form the mucus layer, an important component of the physical gut barrier. Activities such as haemolytic, DNase and gelatinase are also frequently assessed, since these virulence factors have been reported for some bacterial species, including *enterococci* [47].

On this subject, LAB have recently received considerable attention as probiotics since they have "generally regarded as safe" (GRAS) status – apart from their suitable technological properties [12, 49].

# 3. Materials and Methods

## 3.1.    Search Strategy

This study was based on the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement [50].

Studies comprising investigation of assessment of survival of table olives isolates in conditions simulating the GI tract were identified via electronic database search of PubMed, Pubmed Central, Science Direct, Scopus and Web of Science, from the inception of each database to 2 May 2020. Searches were conducted on title, abstract and/or keywords as per the capacity of each database using the following terms: (table olives* OR fermented olives* or traditional olives*) AND (probiotics* OR GI*). Additionally, several reference lists of included studies and available reviews related to table olives were hand searched for additional relevant articles. No language or date limits were applied.

## 3.2.    Eligibility Criteria

To be eligible for the subsequent review, the studies must have accomplished the following criteria: at least one of the tested microorganisms were isolated from table olives; article evaluates the resistance of microorganisms to at least one fraction of the GI tract (mouth, stomach, small intestine and/or large intestine), either by acidic pH, or enzymes/bile tolerance, or both; pH, bile and enzyme concentration, medium used for tests, incubation time and incubation temperature are well described; no language restrictions were imposed; and only studies reported and published in journals in the last ten years were considered.

## 3.3.    Data Extraction

All articles from electronic searches were imported into an Excell library, and any duplicate papers removed. Initial screening was conducted by analyzing titles for relevance, and abstracts with relevant titles were then reviewed for pertinence. Furthermore, every article whose full-text was not available, was further excluded. Finally, full-text manuscripts of potentially eligible studies were obtained and assessed against eligibility criteria.

# 4. Results and Discussion

## 4.1. Eligible studies

According to the search strategy, 720 records were identified, where 430 were duplicates. After the removal of all studies prior to 2010, the remaining 282 papers were screened for titles and abstracts, giving a total of 40 records to be screened for eligibility by full-text review; 4 of those were unfortunately unavailable. After careful full-text screening, 13 articles were rejected due to the reasons listed in Figure 1, and the remaining 23 articles were then entered into the qualitative synthesis procedure.
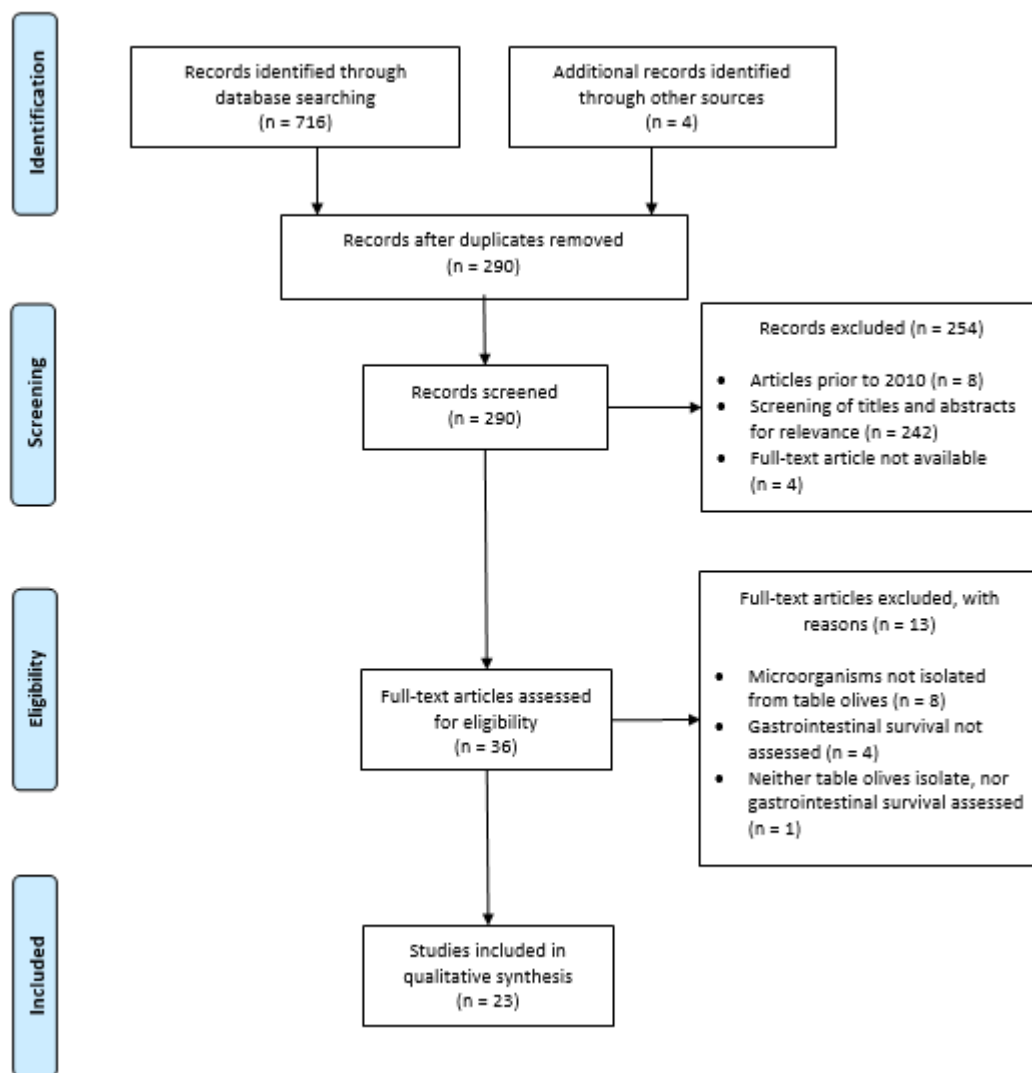


**Figure 2.** PRISMA 2009 flow diagram

## 4.2. Data synthesis

The 23 articles that entered into the qualitative synthesis were divided in groups according to the type of method employed. Some articles report more than one type of method and, therefore, are included in both groups. A synthesis of the methods employed is presented in Table 1.

**Table 1.** Characteristics of the methods reviewed

| Type of digestion | Table olives | Process | Cells State | Simulated digestive juice | pH | Enzymes or Chemical | Digestion time / temperature | Survival Assessment | Year | Ref. |
|---|---|---|---|---|---|---|---|---|---|---|
| Gastric (Stomach) | Moroccan | Natural | Stationary (Overnight) | MRS broth | 3, 2.5 and 2 | Bile salts (3 g/L) | 5 h / 37 ℃ | Plate count after incubation (comparison with control) | 2019 | [51] |
| | Cypriot | Natural | Not described | MRS broth | 6, 4, 3 and 2 | - | 48 h / 37 ℃ | Plate count at 0, 1 and 3 h | 2018 | [52] |
| | Not described | Not described | Stationary (18 h) | PBS | 2.5 | - | 3 h / 37 ℃ | Plate count at 0, 1, 2 and 3 h | 2019 | [53] |
| | Algerian | Not described | Stationary (Overnight) | MRS agar | 3.5 | - | 24 h / 37 ℃ | Visible growth in MRS agar | 2017 | [54] |
| | | | | Bicarbonate buffer | 3 and 2 | Pepsin (3 g/L) | 2 h and 18 h / 37℃ | | | |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Gastric (Stomach)** | Not described | Not described | Stationary (Overnight) | PBS | 3, 2 and 1 / 3 and 2 | - / Pepsin (3 g/L) | 3 h / 37 °C | Plate count at 0, 1 and 3 h | 2016 | [55] |
| | Greek | Natural | Stationary (18 h) | PBS | 2.5 | - | 3 h / 37°C | Plate count at 0, 0.5, 1, 2 and 3 h | 2013 | [20] |
| | Spanish | Natural | Stationary (Overnight) | Saline[a] | 3, 2.5, 2 and 1.5 | Pepsin (3.2 g/L) | 30 min / 37 °C | Plate count before and after incubation | 2012 | [56] |
| | Portuguese | Natural | Not described | YM broth | 2.5 | - | 10 d / 37 °C | Visible growth in YM broth | 2011 | [57] |
| | Italian | Alkalyne | Stationary | MRS broth | 2.5 | - | 24 h / 37 ° C | Plate count at 1, 3, 6, 8 and 24 h (comparison with control) | 2010 | [58] |
| **Intestinal (Small Intestine)** | Cypriot | Natural | Not described | MRS broth | Standard | Bile salts (3 g/L) | 3 h / 37 °C | Plate count at 0 and 3 h | 2018 | [52] |
| | Not described | Not described | Stationary (18 h) | PBS | 8.0 | Bile salts (5 g/L) | 4 h / 37 °C | Plate count at 0, 1, 2, 3 and 4 h | 2019 | [53] |
| | Not described | Not described | Stationary (Overnight) | MRS broth / PBS | Standard / 8.0 | Oxgall bile (3, 5 and 10 g /L) / Pancreatin (1 g/L) | 4 h / 37 °C | Plate count at 0 and 4 h | 2016 | [55] |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Intestinal (Small Intestine)** | Greek | Natural | Stationary (18 h) | PBS | 8.0 | Bile salts (5 g/L) | 4 h / 37ºC | Plate count at 0, 1, 2 and 4 h | 2013 | [20] |
| | Portuguese | Natural | Not described | YNB agar | Standard | Oxgall bile (3 g/L) | 3 d / 27 and 37 ºC | Visible growth in YNB agar | 2011 | [57] |
| **Intestinal (Unspecified Fraction)** | Not described | Not described | Stationary (Overnight) | MRS broth | Standard | Bile salts (0, 3, 6, 18 and 36 g/L) | 24 h /37 ºC | O.D measure at 600 nm after incubation | 2020 | [59] |
| | | | | | | Porcine bile (5 g/L) | | O.D measure at 600 nm every hour during incubation | | |
| | Algerian | Not described | Not described | MRS broth | 4.0 | Bovine bile (3 g/L) | 24 h / 37 ºC | Growth in MRS agar pH 5.8 | 2017 | [54] |
| | | | | | | Bile salts (3 g/L) | 3 h and 24 h / 37ºC | Comparison O.D at 600 nm between MRS with bile and control without bile | | |
| | Spanish | Natural | Stationary (Overnight) | MRS agar | Standard | Bile salts (0-100 g/L with increments of 10 g/L) | 72 h / 37 ºC | Visible growth in MRS agar | 2012 | [56] |
| | Italian | Alkalyne | Stationary | MRS broth | Standard | Bile salts (3 g/L) | 24 h / 37 º C | Plate count at 1, 3, 6, 8 and 24 h (comparison with control) | 2010 | [58] |

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| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **GI (Sequential)** | Spanish | Natural and Alkalyne | (1) Early Stationary | Phosphate buffer | 2.0 | Pepsin (0.0133 g/L) Lysozyme (0.01 g/L) | 2.5 h (~150 rpm) / 37 ℃ | Plate count before and after incubation | 2019 | [60] |
| | | | (2) From gastric digestion | Phosphate buffer | 8.0 | Bile salts (3 g/L) Pancreatin (0.1 g/L) | 3 h (~150 rpm) / 37 ℃ | Plate count after incubation | | |
| | Italian | Natural | (1) Early Stationary | Phosphate buffer | 2.0 | Pepsin (0.0133 g/L) Lysozyme (0.01 g/L) | 2.5 h (~200 rpm) / 37 ℃ | Plate count before and after incubation | 2018 | [61] |
| | | | (2) From gastric digestion | Phosphate buffer | 8.0 | Bile salts (3 g/L) Pancreatin (0.1 g/L) | 3.5 h (~200 rpm) / 37 ℃ | Plate count after incubation | | |
| | Greek | Natural | (1) Stationary (Overnight) | Phosphate buffer | 2.0 | Pepsin (0.0133 g/L) Lysozyme (0.01 g/L) | 2.5 h (~200 rpm) / 37 ℃ | Plate count before and after incubation | 2018 | [14] |
| | | | (2) From gastric digestion | Phosphate buffer | 8.0 | Bile salts (3 g/L) Pancreatin (0.1 g/L) | 3.5 h (~200 rpm) / 37 ℃ | Plate count after incubation | | |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **GI (Sequential)** | Portuguese | Natural | (1) Stationary (24 h) | Phosphate buffer | 2.0 | Pepsin (0.0133 g/L) Lysozyme (0.01 g/L) | 2.5 h (~200 rpm) / 37 ℃ | Plate count before and after incubation | 2017 | [62] |
| | | | (2) From gastric digestion | Phosphate buffer | 8.0 | Bile salts (3 g/L) Pancreatin (0.1 g/L) | 3.5 h (~200 rpm) / 37 ℃ | Plate count after incubation | | |
| | Greek | Natural | (1) Early Stationary | Phosphate buffer | 2.0 | Pepsin (0.0133 g/L) Lysozyme (0.01 g/L) | 2.5 h (~200 rpm) / 37 ℃ | Plate count before and after incubation | 2015 | [63] |
| | | | (2) From gastric digestion | Phosphate buffer | 8.0 | Bile salts (3 g/L) Pancreatin (0.1 g/L) | 3.5 h (~200 rpm) / 37 ℃ | Plate count after incubation | | |
| | Spanish | Natural and Alkalyne | (1) Early Stationary | Phosphate buffer | 2.0 | Pepsin (0.0133 g/L) Lysozyme (0.01 g/L) | 3 h (~150 rpm) / 37 ℃ | Plate count before and after incubation | 2013 | [64] |
| | | | (2) From gastric digestion | Phosphate buffer | 8.0 | Bile salts (3 g/L) Pancreatin (0.1 g/L) | Overnight (150 rpm) / 37 ℃ | Plate count after incubation | | |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **GI (Sequential)** | Italian | Alkalyne | (1) Stationary (24 h) | Phosphate buffer | 2.0 | Pepsin (0.0133 g/L) Lysozyme (0.01 g/L) Porcine bile (0.05 g/L) | 2.5 h (~200 rpm) / 37 ℃ | Plate count before and after incubation | 2014 | [7] |
| | | | (2) From gastric digestion | Phosphate buffer | 8.0 | Bile salts (3 g/L) Pancreatin (0.1 g/L) | 4 h (~200 rpm) / 37 ℃ | Plate count after incubation | | |
| | Not described | Not described | (1) Stationary (Overnight) | Saline[b] | 2.0 | Pepsin (1 g/L) | 2 h (150 rpm) / 37 ℃ | Plate count before and after incubation | 2017 | [65] |
| | | | (2) From gastric digestion | Saline[b] | 7.4 | Bovine bile (5 g/L) Pancreatin (1 g/L) | 3 h / 37 ℃ | Plate count after incubation | | |
| | Spanish | Natural | (1) Stationary (18 h) | PBS | 3.0 | Pepsin (3 g/L) | 3 h / 37 ℃ | Plate count before and after incubation | 2016 | [39] |
| | | | (2) From gastric digestion | PBS | 8.0 | Trypsin (1 g/L) | 8 h / 37 ℃ | Plate count after incubation | | |

| | | | | | pH | | Time / temp | Notes | Year | Ref |
|---|---|---|---|---|---|---|---|---|---|---|
| **GI (Sequential)** | Italian | Alkalyne | (0) Stationary (Overnight) | Bicarbonate buffer | 6.9 | Lysozyme (0.1 g/L) | 5 min / 37 ℃ | - | 2018 | [66] |
| | | | (1) From oral digestion | Bicarbonate buffer | 2.5 | Pepsin (3 g/L) | 1 h / 37 ℃ | Plate count after incubation | | |
| | | | (2) From gastric digestion | Bicarbonate buffer | 7.2 | Bile salts (5 g/L) Pancreatin (1 g/L) | 3 h / 37 ℃ | Plate count at 2 and 3 h | | |
| | Portuguese | Natural | (0) Stationary (Overnight) | Bicarbonate buffer | 6.2 | Lysozyme (0.1 g/L) | - | Plate count before and after addition of lysozyme (G1 and G2) | 2014 | [67] |
| | | | (1) From oral digestion | Bicarbonate buffer | Gradually decreased ( 5 → 4.1→ 3.0 → 2.1 → 1.8) | Pepsin (3 g/L) | 100 min (20 min for each pH, 50 rpm) / 37 ℃ | Plate count after each incubation (G3-G7) | | |
| | | | (2) From gastric digestion (samples after 20, 40 and 60 min of incubation, adjusted to pH 6.5) | Bicarbonate buffer | 8.0 | Bile salts (4.5 g/L) Pancreatin (1 g/L) | 120 min (50 rpm / 37 ℃ | Plate count after incubation (Gi3, Gi4 and Gi5 | | |

| | Portuguese | Not described | (1) Free and microencapsulated | Not described | Gradually decreased ( 5 → 4.1→ 3.0 → 2.1 → 1.8) | Pepsin and Lysozyme (conc. not described) | Total duration of dynamic stress of 160 min | Plate count before and after gastric stress | 2015 | [68] |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | (2) From gastric digestion | Not described | 6.5 | Bile salts and pancreatin (conc. not described) | | Plate count after intestinal stress | | |
| **GI (Sequential)** | Spanish | Alkalyne | (1) | Food matrix (olive)[c] | 2 → 6 → 5.7 → 4.5 → 2.9 → 2.3 → 1.8 → 1.6 | 0.25 ml/min of pepsin (2080 IU/ml) 0.25 ml/min of lípase (250.5 IU/ml) | Total duration of 5 h | Plate count before its introduction into the artificial stomach, and regularly collected during digestion in the different compartments of the system (stomach, duodenum, jejunum, and ileum) | 2014 | [26]* |
| | | | (2.1) | Food matrix (olive)[c] | 6.0 | 0.5 ml/min of bile salts (4% during the first 30 min of digestion and then 2%), 0.25 ml/min of pancreatic juice (103 USP/ml), 0.25 ml/min of intestinal electrolyte solution, trypsin (23,600 IU) | | | | |

| | | | |
|---|---|---|---|
| (2.2) | Food matrix (olive)[c] | 6.9 | - |
| (2.3) | Food matrix (olive)[c] | 7.2 | - |

Notes: (0) – oral phase; (1) – gastric phase; (2) – intestinal phase; (2.1) – duodenum; (2.2) – jejunum; (2.3) – ileum

*Dynamic multi-compartimental *in vitro* model, denominated TIM system, completely automatized.

[a]; Saline: 0.2% NaCl

[b]; Saline: concentration not described

[c]: Food olive matrix: 100 g pasteurized olives/L of sterile water

## 4.3.    Mouth Resistance

The mouth is the first organ of the GI tract, and represents the beginning of the digestive process of food. Inside the mouth, chewing breaks the food into pieces that are more easily digested. At the same time, saliva mixes with food to begin the process of breaking it down into a form that the body can absorb and use, by transforming it into a smooth bolus that can be swallowed down the esophagus to enter the stomach [69]. Saliva is released by the salivary glands, and contains digestive enzymes, such as amylase and lipase, that are responsible for breaking starch into simple sugars and for breaking fats, respectively [69, 70]. In addition, saliva is constituted by lysozyme, an enzyme widely used in the preservation of foods. Lysozyme exhibits antimicrobial activity, once it disrupts formation of the major component of the gram-positive bacterial cell wall (peptidoglycan) – by breaking 1,4-beta-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues [71]. The hydrolysis of peptidoglycans may indeed compromise the integrity of bacterial cell walls, thus leading to lysis of the bacteria [71, 72]. Therefore, a potential probiotic must sustain the hostile agents present in the mouth, particularly in saliva at the moment it is ingested within the food, in order to achieve the gut while viable.

### 4.3.1.Review of methods

The assessment of resistance of indigenous microbiota from table olives to the conditions experienced in the mouth was reported in two articles. In both cases, the main feature evaluated was the tolerance of the microorganisms isolated to the antimicrobial action of the enzyme lysozyme. In a study by Peres et al. [67] single lactobacilli cultures isolated from natural fermented table olives of Galega cultivar, previously propagated in 35 mL of MRS broth were harvested by centrifugation ($3214 \times g$, 10 min, 10 ºC). The pellet was then resuspended in 5 mL of an electrolyte solution (6.2 g/L NaCl, 2.2 g/L KCl, 0.22 g/L $CaCl_2$ and 1.2 g/L $NaHCO_3$, pH 6.2), functioning as a simulated salivary juice (SSJ),  and added with 5 mL of eletrolyte solution containing lyzozyme to a final concentration of 0.01 g/L. The tolerance was evaluated through assessement of survival and consequent growth by inoculation on MRS agar. Guantario et al. [66] simulated saliva using an eletrolyte solution with the same composition, yet with a pH of 6.9. The microorganisms isolated from Italian natural fermentation table olives, Nocellara del Belice, were propagated overnight until a stationary state. A volume of 3 mL of cell culture was also harvested by centrifugation ($5,000 \times g$, 15min, 4 ºC) and ressuspended in the same amount of the simulating salivary juice, for 5 minutes at 37 ºC.

Survival was not assessed, and instead the cell culture was transferred, after incubation, to the gastric phase of GI simulation.

### 4.3.2. Critical assessment of analytical methods

As previously stated, the environment felt in the mouth, particularly the action of saliva, may exert a negative effect upon microorganisms carried by food that might otherwise exhibit health benefits to the host – as cell wall destruction carried out by lysozyme, for example. Despite the importance, due to those facts, of assessing the ability of microbiota to survive the very first part of food digestion, only two authors among 23 took it into account when producing a protocol to evaluate GI tract resistance.

The normal value of pH of saliva for a healthy human varies between 6.2 and 7.6. A major distinction between the two protocols is the pH value of the solution simulating salivary juice, since Peres et al. [67] considers a value of 6.2 which matches the most acidic value, while Guantario et al. [66] uses the average value of 6.9. Although both values are appliable and in agreement with *in vivo* conditions, evaluating resistance using a lower pH may lead to more reliable results and, therefore, conclusions – since it assures that microorganisms are tested in the most harsh conditions that can be faced in the mouth; this corresponds to higher inhibition at more acidic pH values, and enhanced activity of lysozyme (optimum pH = 6.2) [73]. The concentrations of enzyme used in both reports are in the range found for normal levels in saliva, and thus matches *in vivo* situations (0.049-0.182 g/L) [71, 74].

The period of action of the oral phase of digestion varied between no incubation and 5 minutes of incubation, on account of the two papers. Although an oral digestion time of 5 minutes has been recommended in order to ensure proper mechanical action for static models, chewing time *in vivo* is generally much shorter; hence, a period of contact with lysozyme of 2 minutes may be more in agreement with what happens in reality [75, 76].

The method used for assessing survival after mouth digestion is the pour-plating method with subsequent enumeration, since it is the most common and accurate method for determining the total culturable count – and is frequently used to assess the total viable count [77]. Despite considering the digestion phase of the mouth, Guantario et al. [66] did not evaluate the survival upon it, which prevents withdrawal of conclusions.

### 4.3.3. Microorganisms tested

Both studies focused on LAB isolates, evaluating bacterial strains belonging to several genera and species, such as *L. plantarum, Lactobacillus paraplantarum, L. pentosus,*

*Lactobacillus coryniformis, Lactobacillus oligofermentens, Leuconostoc mesenteroides, Enterococcus casseliflavus* and *Enterococcus gallinarum*.

## 4.4.     Stomach/Gastric Resistance

### 4.4.1. Context

After ingestion in the mouth, the bolus produced is swallowed down the esophagus to enter the stomach, where the gastric phase of digestion takes place, with segregation of gastric juice. Approximately 2.5 L of gastric juice is secreted into the human digestive tract every day [78]. In the stomach, the environment undergoes several changes, as an extreme reduction in pH. The pH value inside the human stomach gradually drops to values around 1.5-3.5, across gastric digestion, due to the secretion of hydrochloric acid by the parietal cells of the stomach [22, 36]. Highly acidic conditions often exert harmful effects in most microorganims, such as denaturation of proteins and loss of viability, so it is crucial to test low pH tolerance for the selection of potential probiotics; this will ensure their ability to adapt to low pH through a mechanism called acid tolerance response and, therefore, resist passage through the stomach during digestion [24, 37, 40]. Furthermore, gastric juice is rich in digestive enzymes, such as pepsin and gastric lipase, which may also cause inhibition towards ingested microbiota [79].

### 4.4.2. Review of methods

Gastric condition tolerance is a crucial parameter for probiotic strain selection. The adaptability to the gastric environment was studied in nine articles. For the vast majority of isolates tested, an initial step of inoculation was performed in order to reach a stationary phase, by incubation for 18 hours [20, 53] or overnight [21, 54-56]. After harvesting of cells, different paths were followed in the protocol to assess gastric survival, starting immediately with the type of solution used to mimic the environment in the stomach. Several authors chose to maintain the culture medium in which microorganisms have previously been grown, modified by reducing the pH and/or adding substances typically found in gastric juice, specifically MRS broth [21, 52, 58] or YM broth, depending on testing LAB or yeasts. Conversely, other authors opted for phosphate buffer saline (PBS) [20, 53, 55]. Among the eight papers, several differences are observed both on pH values and incubation times tested, as methods to assess survival. All authors reported a temperature of 37 ºC during the gastric simulation.

Bevilacqua et al. [58] investigated resistance resorting to the inoculation of isolates onto MRS broth acidified to pH 2.5; survival was assessed in MRS agar after 1, 3, 6, 8 and

24 hours of incubation. The same medium was used by Anagnostopoulos et al. [52], but buffered by a different range of pH (6.0, 4.0, 3.0 and 2.0) and survival was assessed only at 1 and 3 hours of incubation. Despite using MRS broth adjusted to different pH values (3.0, 2.5 and 2.0) to simulate gastric conditions, Abouloifa et al. [21] modified the method via addition of bile salts and incubation for a total of 5 hours. Since Silva et al. [57] worked with yeasts, the medium used to simulate gastric conditions was Yeast Malt (YM) broth, also modified to pH 2.5. However, the incubation time, unlike the previous ones (which was just a few hours as happens during digestion), was up to 10 days, and survival was assessed by observing the presence or not of growth within the broth.

Abriouel et al. [56], tested, in turn, the resistance to passage in the gastric tract by transferring 1 mL of overnight cell culture to 19 mL of a saline-based broth (2 g/L NaCL), adjusted to different pH values (1.5, 2.0, 2.5 and 3.0), but with addition of the digestive enzyme pepsin (3.2 g/L), and incubation for 30 minutes

Both Argyri et al. [20] and Pavli et al. [53] proposed a quite similar method, evaluating low pH tolerance by suspending their isolates in a PBS solution adjusted to pH 2.5, without addition of any gastric enzyme, following incubation of 3 h. Tolerance was assessed after 1, 2 and 3 hours of incubation in both reports, and at 0.5 h of incubation in Argyri et al. [20] by the plating technique.

Taheur et al. [55] on the other hand, investigated the resistance to simulated gastric conditions using two distinct protocols, analysing either low pH tolerance or pepsin tolerance. On the one hand, isolated microorganisms were suspended in a PBS solution with pH values adjusted to 3.0, 2.0 and 1.0. On the other hand, PBS was supplemented with pepsin (3 g/L), and the pH adjusted 3.0 or 2.0. Both inocula were incubated for 3 hours, and survival was assessed at the 1st and 3rd hours of incubation, by enumeration on MRS agar.

Mermouri et al. [54] subjected microbial isolates to gastric conditions as well, by both acid and pepsin stresses; however, the methods employed showed significant differences from other reports. For low pH resistance, harvested cells were washed in PBS pH 7.4 and directly spotted onto an MRS agar plate, previously acidified to pH 3.5, rather than using a fluid mimicking gastric juice. Viability was assessed after 24 hours of incubation. Alternatively, broth (NaCl 125 Mm/L, KCl 7 Mm/L, $NaHCO_3$ 45 Mm/L) was used with a composition similar to the gastric fluid segregated in the stomach, supplemented with pepsin (3 g/L) and adjusted to pH values 3.0 and 2.0 – to test the capacity of bacteria to grow, within the broth, after incubation for 2 and 18 hours.

### 4.4.3. Critical assessment of analytical methods

The environment inside a human stomach can be extremely severe for the majority of microorganisms that populate ingested food, mainly due to segregation of gastric juice, as previously noted. Therefore, the choice of medium in which the tolerance to gastric digestion is assessed is of relevance, in order to obtain results as reliable as possible. Gastric juice consists of an eletrolyte solution composed mostly by sodium chloride, potassium chloride and hydrochloric acid. Several authors opted to use the selective growth medium, modified to match the pH as per gastric conditions – particularly MRS broth or YM broth, depending on testing bacteria or yeasts, respectively. Both media are designed to favour abundant growth of bacteria and yeasts, providing large amounts of sources of carbon (glucose, in this case), nitrogen and vitamins, unlike gastric juice. Hence, the presence of these growth factors might act as a protective effect onto the isolates, influencing positively their ability to tolerate the simulated gastric environment and, consequently, display outcomes that deviate from reality [80]. The same rationale goes for the direct inoculation onto MRS agar acidified, as done by Mermouri et al. [54]. This procedure leads to inability to define a period of incubation, since at least 24 hours is required for bacteria to grow, as well as to take samples before and after certain times of incubation – and, therefore, to assess the percent survival by different times of incubation. On the contrary, PBS consists of water-based salt solution containing disodium hydrogen phosphate and sodium chloride; it aids in maintaining a constant pH, thus making it a good solution for use in biological research. As it does neither contribute to the growth of microbiota, given the absence of an energy source, nor does it harm the cells, its utilization might be more appropriate than growth media. Another solution, presented in a single study, was the production of a synthetic gastric juice, an eletrolyte solution with a composition similar to the one found *in vivo*; this could theoretically lead to a more trustworthy simulation. Although PBS and a synthetic gastric juice seem to be good choices for applied studies, previous studies had reported survival of lactobacilli slightly lower when PBS was used rather than gastric juice – since components in the gastric juice may confer some protective effect upon the bacterial cell. This fact suggests the use of PBS at the desired pH to screen strains for their ability to maintain viability *in vivo*, when exposed to gastric juice [81].

As pH dramatically varies in the stomach between the beginning and the end of digestion, the value or range of values tested are of extreme importance to evaluate the actual resistance to GI tract passage. At the moment of food intake, pH is ca. 5, but the secretion of hydrochloric acid lowers it to the values of optimal enzyme activities. By the end of stomach digestion, pH may be below 2.0 [76]. Although Abouloifa et al. [21]

did test pH values ranging between 6.0 and 2.0, it may be unsuitable to assess tolerance against pH above 4 – since such high levels of pH are experienced only at the beginning of digestion, and it varies between 3.5 and 1.5 throughout most of digestion. Furthermore, performing the tests using lower values of pH promotes selection of more acid-tolerant strains. Due to emptying of the stomach during digestion, most of the strains already passed into the duodenum, when pH reaches its minimum, under 2.0. In addition, the probiotics strains could be buffered by food or other carrier matrices with which they are ingested and, therefore, not be exposed to the current pH of the stomach [82, 83]. Therefore, the pH should represent a mean value for a general meal [76] for the sake of gastric simulation. For example, for a study of standardized static *in vitro* digestion considering food, a static value of pH 3.0 is recommended [76]. Other studies suggest a pH value of 2.5; even though it is not the most common pH value in the human stomach, it assures isolation of the very acid-tolerant strains [84]. A more extreme pH value of 2.0 could also be taken into account; considering that the stomach is almost empty when the pH is under 2.0, tolerance could be assessed for a range of pH values between 2.0 and 3.0.

Most papers evaluated only the effect of the acid environment prevailing in the stomach, yet gastric juice also carries digestive enzymes – with activity favoured and even reaching its maximum due to such an acidity [76]. Since the main enzyme segregated is pepsin, assessing its effect together with low values of pH may lead to different results pertaining to the capacity of potential probiotics to reach the human gut, regarding intolerance towards the action of the enzyme. During digestion, pepsin amount increases from 0.26 to 0.58 g/L, and significant variations on its activity are reported in several studies [76, 85–87]. Hence, the use of pepsin at 2000 U/mL in the final digestion mixture is recommended taking into account the values reported in the literature, whereby its final concentration depends on the quality of the enzyme [76]. Although bile acids may also be segregated during the gastric phase of digestion, only low levels can be found - and not in all individuals, whereas its inclusion on an *in vitro* gastric digestion model is not seen as crucial.

Another point of divergence between the nine papers encompasses the period of incubation, during the simulation of gastric digestion – varying between 30 minutes and 10 days. Within the stomach, digestion and emptying of a solid meal is usually completed between 2 and 4 hours, depending on the type of food and the individual [84]. Therefore, assessing the survival of isolates after a time of incubation greater than 5 hours, as performed by several authors, does not necessarily allow accurate conclusions regarding their resistance. Furthermore, an assay time of 10 days is exceedingly superior than the average digestion time, thereby leading to an underestimation of the strains able to

endure the gastric conditions during digestion; while a period of 30 minutes may lead to the opposite outcome. An average value of 3 hours is frequently employed when simulating gastric digestion, since it corresponds to almost complete emptying of the stomach for every kind of meal. From other perspectives, 2 hours of digestion is suggested, applicable to a broad range of meals, because it represents the half emptying of a moderately nutritious and semi-solid meal [76]. Therefore, an incubation period of between 2 and 3 hours is necessary for a reliable assessment of resistance, compared to an *in vivo* situation.

The method for assessing survival is crucial to understand and draw conclusions about the capacity of a chosen strain to exhibit probiotic traits, since the possible health benefits only exist if a high number of viable cells (at least $10^6$-$10^7$) reach the human gut [88, 89]. Hence, it is crucial to determine the decrease in viability before and after gastric simulation - to ensure not only that the strain is still able to grow, but also that most cells survived. The great majority of the papers applied the pour-plating technique and, consequently, enumerated the viable colonies, thus allowing a quantitative comparison before and after the simulation. Still, a minority of the reports evaluated the tolerance solely through a qualitative assessment of the capacity of a strain to grow on a broth or agar simulating gastric conditions. Since a qualitative evaluation only permits conclusion on whether there is still growth or the gastric conditions completely inhibit the strain, it does not give enough information to conclude on how much tolerance a selected strain will exhibit.

### 4.4.4. Microorganisms tested

Among the several reports analyzed, a diversified range of microbiota was isolated and tested, from bacteria to yeasts (see Table 2).

**Table 2.** Types of microorganisms tested for resistance to gastric conditions

| Lactic acid bacteria | Yeast | Reference |
|:---:|:---:|:---:|
| + | | Abouloifa et al. (2019)[21] |
| + | | Pavli et al. (2019)[53] |
| + | | Anagnostopoulos et al. (2018)[52] |
| + | | Mermouri et al. (2017)[54] |
| + | | Taheur et al. (2016)[55] |
| + | | Argyri et al. (2013)[90] |
| + | | Abriouel et al. (2012)[56] |
| | + | Silva et al. (2011)[67] |
| + | | Bevilacqua et al. (2010)[58] |

Bacterial strains belonged to the following LAB genera and species: *L. plantarum, L. pentosus, Lactobacillus brevis, L. paraplantarum, L. coryniformis, Lactobacillus paracasei, Ln mesenteroides, Leuconostoc pseudomesenteroides, Pediococcus ethanolidurans, Pediococcus parvulus, Enterococcus faecium* and *Enterococcus faecalis*. Several species of yeasts were also tested, such as *Candida boidinii, Candida oleophila, Candida citrea, Candida sake, Candida silvae, Candida valida, Candida norvegica, Citeromyces matritensis, Pichia membranaefaciens, Pichia fermentans, Metschnikovia pulcherrima, Rhodosporidium capitatum, Torulaspora delbrueckii, Trichosporum pullulans, Saccharomyces cerevisiae* and *Kloeckera apiculata*.

## 4.5.    Intestine Resistance

### 4.5.1. Context

Chyme, partially digested food provided from the stomach, and the attached microbiota enter in the upper part of the intestine, denominated small intestine. Here

many substances are segregated, such as bile, pancreatic enzymes (trypsin, chymotrypsin, amylase, and lipase), and bicarbonates, leading to a pH increase [91]. The small intestine is divided into three fractions, denominated duodenum, jejunum and ileum, from the upper to the lower part of the organ. As the chyme goes down the small intestine, the concentration of bile and pancreatic enzymes decrease, and the medium becomes more neutral [28, 91, 92]. The large intestine is characterized by an even more diluted bile concentration and a higher value of pH, which corresponds to a more favorable environment for microbial colonization [91].

Bile is a digestive secretion synthesized from cholesterol, mainly composed by water, bile acids, biliverdin, and phospholipids; it plays an essential role in the emulsification and solubilization of lipids, as well as in specific and non-specific defense mechanism of the gut [59, 93, 94]. Being highly hydrophobic and at high levels, specific bile acid (BA) moieties, due to their detergent properties, exhibit antibacterial effects; they indeed affect the phospholipids and proteins of cell membranes and disrupt cellular homeostasis, or can even cause oxidative damage to DNA [93, 94]. Hence, the ability of microbes to tolerate bile and BAs is recognised necessary for their survival and their persistence in the GI tract, particularly in the intestinal fraction – where segregation of this substance is more relevant and to higher levels.

### 4.5.2. Review of methods

A total of nine papers investigated the individual resistance of microbiota from table olives to intestinal conditions. Among them, some specify the intestinal fraction whose conditions were tested, which in this case was always the small intestine or some small intestine fraction. . Conversely, others do not specify it and evaluate tolerance to conditions that would allow the passage and survival of microbiota through the intestine in general. As for the methods of gastric resistance, an initial step of inoculation is described for the great majority of isolates tested, to reach a culture in stationary phase [20, 53, 55, 58, 59]. All the models for simulation of intestinal conditions reported 37 ℃ as the temperature at which the tests should be performed.

### 4.5.2.1. Small intestine

Harvested microbiota was assessed for its tolerance to the small intestine environment. Protocols differ from each other in some factors, such as pH, bile concentration, intestinal/pancreatic enzymes presence and concentration, medium where tests are carried out, and reaction time. For all studies, the survival of the strains

was assessed by the enumeration of viable colonies formed (MRS agar for bacteria and YNB agar for yeasts).

MRS broth was used by Anagnostopoulos et al. [52] as the fluid to mimic the secretions that occur inside the small intestine. The medium was modified by the addition of bile acids to a concentration of 3 g/L; however, no change to the standard pH value ($\approx 6.2$) has been described. The intestinal conditions were simulated for 3 hours, and tolerance was assessed comparing the survival before and after incubation.

As seen for gastric simulation, both Argyri et al. [20] and Pavli et al. [53] followed the same protocol to check the capacity to survive in the small intestine, by suspending the isolate cells in a PBS solution (pH 8.0), with bile salts (5 g/L), followed by 4 hours of incubation – thus reflecting the time spent by food in the digestive organ. The two methods differ from each other in terms of the periods of assessment of resistance by enumeration of viable colonies in MRS agar. The former evaluates survival on samples before incubation and after 1, 2 and 4 hours of incubation, and the latter takes an aliquot from the suspension at the 3rd hour of incubation as well.

Taheur et al. [55] evaluated the tolerance to the action of bile and to the effect of pancreatin in two separate assays. The former was assessed in MRS broth supplemented with different concentrations of bovine bile (3, 5 and 10 g/L), while the latter used a PBS solution adjusted to pH 8.0 and supplemented with pancreatin (1 g/L). Both assays have considered 4 hours of enteric simulation.

A different approach was performed by Silva et al. [57] since the ability of the strains to grow in the presence of bile-like conditions analogous to those prevailing in the duodenum was evaluated by direct inoculation in YNB agar plates containing ox gall bile (3 g/L). On the other hand, the period of incubation (10 days) was significantly larger, compared to the other methods, and incubation was performed both at 27 and 37 °C.

### 4.5.2.2. Unspecified intestinal fraction

In the case of Bevilacqua et al. [58], the medium (MRS broth) was added with bile salts to a concentration of 3 g/L, and tolerance was assessed by plate count in MRS agar after 1, 3, 6, 8 and 24 hours of incubation. Alternatively, Abriouel et al. [56] spotted the bacterial culture onto MRS agar supplemented with different concentrations of bile salts, between 0 and 100 g/L, with increments of 10 g/L. Resistance was examined through the ability of the strain to grow in the presence of different concentrations of bile, by 72 hours of incubation.

The remaining two papers studied the tolerance not only to bile salts, but also to the effect of the whole bile, separately. Prete et al. [59], studied bile salts resistance

by suspending the cell culture ($10^3$ CFU/mL) in the medium with increasing concentration of the substance (0, 3, 6, 18 and 36 g/L). In contrast, the resistance to whole bile was evaluated by inoculation in MRS broth added with porcine bile (5 g/L). Both assays entailed a period of incubation of 24 hours, and resistance was assessed by a spectrophotometric method – in which microbial growth was controlled by measuring optical density at 600 nm after 24 hours for bile salts, and every hour during incubation for whole porcine bile. In the case of Mermouri et al. [54], bacteria were inoculated in MRS broth acidified to pH 4.0, supplemented with bovine bile or bile salt, at a concentration of 3 g/L. For the former method, the suspension was incubated for 24 hours and then spotted on MRS agar pH 5.8 to evaluate tolerance by checking for growth. In the second method, incubation periods of 3 and 24 hours were tested, and, at the same time, the isolates were re-suspended in MRS broth, without bile, pH 5.8, to evaluate the survival percent by comparing optical density at 600 nm of both suspensions.

### 4.5.3. Critical assessment of analytical methods

Similarly to what was observed in the methods for simulation of gastric digestion, the reports on simulation of intestinal conditions tendentially use MRS broth or PBS as an artificial intestinal juice. As discussed previously, the abundant sources of energy and growth factors present in growth media as MRS may impart microorganisms a higher resistance. Consequently, the ability to grow in environments rich in bile salts – which would otherwise be lethal or at least a bit inhibitory for them (even taking into account that the isolates are carried within food) may still provide some energy sources for them [80]. Although inoculation in YNB agar supplemented with bile may allow assessment of the ability of the isolates to grow in the presence of the compound, it does not allow conclusions on the antimicrobial effect that bile may have during the period of digestion. Once when simulation is performed on a broth and samples can be withdrawn after different periods of incubation, the isolates grow in this case in its presence for 3 whole days, thus translating into a qualitative result [63]. Therefore, the use of PBS as the broth simulating intestinal segregations seems to be a safer option to achieve reliable results, comparable to *in vivo* situations. Another hypothesis, although not contemplated by the reviewed papers, is the preparation of an artificial intestinal juice.

Although a portion of the methods does not specify the fraction of the intestine whose environment is tested, in general all methods evaluate the conditions, essentially levels of bile, that are found in the small intestine – which are known to be more harmful to microbiota [57]. The large intestine environment is not taken into account when assessing the ability of a strain to survive passage in the GI tract. Probably the organ has

an overriding function of absorption, instead of degradation, so the concentration of bile and other enzymes is significantly lower, which favours abundant growth and colonization by bacteria [91].

The most critical factor in the capacity of microbiota to transit and colonize the intestinal tract is their resistance to the detergent action of bile; this is where a more considerable variation is observed in the methods employed. Once bile acids are the main responsible for the antibacterial effect of bile, several authors tested the resistance against bile acids instead of whole bile. However, the *in vivo* antibacterial activity of bile may be lower than observed in broth systems as bile salts complexed in micelles with phospholipids. The majority of bile acids present in the small bowel may not be free to interact with bacterial cells, whereby testing bile resistance with whole bile extract should lead to a better simulation of intestinal digestion [59, 92, 94]. The whole bile extracts are frequently either from bovine or porcine origin; although bovine bile is commonly chosen to assess the *in vitro* bile tolerance of bacterial strains, porcine bile is more similar to human bile concerning bile salt/cholesterol, phospholipid/cholesterol and glycine to taurine ratios and is more inhibitory [54, 76, 94]. Despite its importance in selecting strains with probiotic potential, bile concentration varies largely in the intestine – and there is no consensus about the precise concentration to which the selected strain should be tolerant [36, 92]. A mean value frequently believed for bile concentration is 3 g/L, considered as critical and sufficient to screen for bile tolerance and resistance [28]

In comparison, other studies consider a concentration of 5 g/L, which is still within the range of values found for bile in the intestine [35, 36, 95]. Bile acid levels are also relatively low until ingestion of a fatty meal [94]. Therefore, a value between these concentrations should be enough to assess survival in the intestinal tract.

Although only tested in a single paper, pancreatic enzymes are some of the most important components in small intestine digestion – and are thus also an important component when assessing the resistance to the intestinal tract [96]. Therefore, the simulated pancreatic/intestinal juice should be constituted by both bile and pancreatic enzymes. The solution could be supplemented either with individual enzymes or porcine pancreatin that contains all the critical pancreatic enzymes but in differing amounts [76]. Pancreatin is usually employed on *in vitro* tests for simulation of intestinal digestion at a concentration of 0.1 g/L [83, 96]. However, higher levels (1 g/L) were reported by Taheur et al. [55] and other authors.

Some variations are found in the pH value across the different fractions of the intestine, as it becomes higher as you go down the organ [91]. As the chyme, after stomach emptying, is neutralized through the secretion of carbonate, the pH on the

upper part of the small intestine (duodenum) is around 6.5 and it increases to about 7.5 in the distal ileum [76]. A simulated intestinal juice (SIJ) with a pH of 8.0, as used in several of the studies reviewed, is just a bit above this range - and, therefore, may as well simulate conditions similar to those of the intestinal tract. Similarly, the utilization of a growth medium without previous pH adjustments may also be valid, since it has a pH just a little more acidic than 6.5. Nevertheless, an average value of 7.0 is recommended to mimic the pH in the entire passage through the small intestinal phase in static conditions [75, 97]. Conversely, a pH of 4.0 is way under of that felt in the upper part of the small intestine; for that reason, is not appropriate when assessing survival on the passage in the gut.

Periods of incubation in the presence of bile and/or pancreatic enzymes varied between 3 and 72 hours. During digestion, the transit time through the small intestine is affected by many variables, and is usually around 3-4 hours [98, 99]. Moreover, the duodenum corresponds to the small intestinal fraction with higher levels of bile and enzymes and lower pH; therefore, it exhibits most potential to inhibit microorganisms, and a shorter period of incubation, simulating the passage of the chyme through that intestinal fraction, could also be considered. Simulated intestinal digestion lasting for a time between 2 and 4 hours may indeed be necessary for a realistic evaluation of resistance through the intestinal tract [76].

Similarly to the stomach, the assessment of survival was chiefly performed by enumeration on an agar plate, which allows quantification of the number of viable cells after a certain period of incubation and comparison with the viability the same strain exhibits without withstanding the intestinal simulation. As described before, it is essential to assess the log reduction and the population of microbiota at the end of intestinal digestion, since the possible health benefits of a probiotic strain are enhanced if a high number of cells remain viable in the intestinal tract. The spectrophotometric methods performed by Prete et al. [59] and Mermouri et al. [54] allow calculation of the influence of the intestinal environment on the capacity to grow and proliferate of the strains by turbidimetrically – comparing the growth in a broth with bile and another without it; however, they do not refer to the number of cells viable by the end of the assay. Even if a strain is not able to grow in the presence of bile, if it retains its viability (or the number of viable cells decreases little, so that enough are left to exert their effect), then it exhibits resistance to the passage in the intestinal tract. Finally, although this turbidimetric method may be faster than plate count, it is applicable only within a certain concentration range [100].

### 4.5.4. Microorganisms tested

Microbiota from bacteria and yeast domains was isolated and tested (see Table 3).

**Table 3.** Types of microorganisms tested for resistance to intestinal conditions

| Lactic acid bacteria | Yeast | Reference |
|:---:|:---:|:---:|
| + | | Prete et al. (2020)[59] |
| + | | Pavli et al. (2019)[53] |
| + | | Anagnostopoulos et al. (2018)[52] |
| + | | Mermouri et al. (2017)[54] |
| + | | Taheur et al. (2016)[55] |
| + | | Argyri et al. (2013)[90] |
| + | | Abriouel et al. (2012)[56] |
| | + | Silva et al. (2011)[67] |
| + | | Bevilacqua et al. (2010)[58] |

The bacterial strains belonged to several LAB genera and species particularly *L. plantarum, L. pentosus, L. paraplantarum, L. coryniformis, L. paracasei, Ln mesenteroides, Ln. pseudomesenteroides, Pd. ethanolidurans, Pd. parvulus, E. faecium* and *E. faecalis*. Several species of yeasts were also tested, such as *C. boidinii, C. oleophila, C. citrea, C. sake, C. silvae, C. valida, C. norvegica, Ct. matriensis, P. membranaefaciens, P. fermentans, M. pulcherrima, R. capitatum, T. delbrueckii, Tr. pullulans, S. cerevisiae* and *K. apiculata*.

## 4.6. Sequential GI Tract Resistance

### 4.6.1. Context

*In vivo* digestion consists in a sequential passage of the food through successively changing environments – starting from the mouth until reaching the intestine, and passing through the stomach halfway [92]. Although the effect of low pH in the stomach, and the action of bile in the gut have extreme relevance when choosing microorganisms for their potential as probiotics, evaluating the behaviour of strains to each component in

separate experiments may result in unrealistic outcomes - and therefore lead to a good partial choice but inappropriate global selection This is due to the possibility that successive stresses, as gastric acid and bile, may interact and thereby exhibit a synergistic effect upon viability of the strains, which may entail a stronger antimicrobial action than either of the stresses alone [35, 92, 96]. In fact, it has been proposed that damaged microorganisms may have different and unpredictable responses to new stress factors, such as an increased susceptibility of bacterial cells to bile action after exposure to a low pH environment [92, 101]. Therefore, several *in vitro* models which sequentially simulate the conditions of different sections of the human GI tract, have been developed to study the survival rate of potential probiotic strains.

### 4.6.2. Review of methods

*In vitro* methods for the evaluation of resistance of potential probiotics, from olive microbiota, to the sequential stresses experienced in the GI tract during food digestion, rather than independent assessments for each section of the tract, were reported in thirteen papers. The majority of the reports described static models, where the conditions on each phase of the artificial digestion remained essentially the same. Except for one paper [68], where it was not described, a basic step of propagation of cells in order for them to achieve a stationary phase was performed. Several studies considered an initial concentration of the inoculum for the beginning of the assays, of $10^7$ [14, 61–63], $10^8$ [68], $10^9$ [80] or between $10^7$ and $10^{10}$ CFU/mL [60, 64]. All assays were performed at 37 ℃, thus mimicking the temperature found in the human body and survival assessment was accomplished by enumeration in agar plate, after each digestion phase.

Seven papers described the use of an electrolyte solution/buffer (2.05 g/L NaCl, 0.60 g/L $KH_2PO_4$, 0.11 g/L $CaCl_2$ and 0.37 g/L KCl) for the simulation of gastric juice, and another buffer consisting of sodium phosphate dibasic heptahydrate and NaCl for the simulation of pancreatic juice, when attempting to simulate GI conditions. The same method was followed in four different articles [14, 61–63]. In the phase of gastric digestion, cells were suspended, at a concentration of about 7 $\log_{10}$ CFU/mL, in simulated gastric juice (SGJ), to evaluate their susceptibility at pH 2.0 and to the presence of pepsin (0.0133 g/L) and lysozyme (0.01 g/L), and incubated for 2.5 hours. The cells were, then, centrifuged washed and resuspended in SIJ, at pH 8.0 and containing bile salts (3.0 g/L) and pancreatin (0.1 g/L), for 3.5 hours. Both Bautista-Gallego et al. [64] and Benítez-Cabello et al. [60] also performed similar methods; however, the duration of the pancreatic digestion differed, being overnight and 3 hours, respectively. Besides, Botta

et al. [7] supplemented the synthetic gastric juice with porcine bile (0.05 g/L), and the duration of gastric and pancreatic simulations were 2 and 4 hours, respectively. Incubations took place in an orbital shaker, to simulate peristaltic movements.

Prete et al. [65] did not describe the composition of the artificial GI juices employed; they simulated gastric conditions in the presence of 1 g/L of pepsin at pH 2.0 for 2 hours, followed by a simulation of intestinal conditions at pH 7.4, in the presence of bovine bile (5 g/L) and pancreatin (1 g/L) for 3 hours.

In a slightly different way, Montoro et al. [39] simulated transit tolerance using a PBS solution intended to parallel GI juices. For gastric digestion, PBS was supplemented with pepsin (3 g/L) and adjusted to pH 3.0. At the same time, in the subsequent intestinal phase, cells were suspended in PBS supplemented with trypsin (1 g/L) and adjusted to pH 8.0. The survival of the isolates was assessed at 1, 2, and 3 hours and 2, 4, and 8 hours of incubation in the gastric and in the intestinal environment, respectively. Besides the effect of pH and digestive enzymes, the effect of the nitrate (5 mM) and glucose (500 mM) was also studied in both simulated gastric conditions.

Still performing a static method, Guantario et al. [66] divided the GI tolerance assay into three compartments - oral, gastric and intestinal. After the oral digestion (reviewed in the section 4.2.2), the gastric digestion was performed at pH 2.5 in the presence of pepsin (3 g/L) for 1 hour and then the sample was transferred to SIJ with bile salts (5 g/L) and pancreatin (1 g/L) for 2 or 3 hours.

A dynamic method was performed by Peres et al. [67], where the emptying of the stomach at increasingly lower pH and transit time of food through the stomach were taken into account. After the simulation of *in vivo* saliva conditions (as described in section 4.2.2), 3 mL of electrolyte solution containing pepsin (3 g/L) to simulate gastric environment was added to the cell suspension, and the pH curve in the stomach was reproduced – starting at 5.0, and gradually decreasing to 4.1, 3.0, 2.1 and 1.8, whereas the gastric emptying was simulated by removing aliquots after sequential incubations of 20 minutes at each pH, in an orbital shaker. The samples withdrawn after incubation at pH values between 5.0 and 3.0 were fed for intestinal simulation - via addition of 4 mL of electrolyte solution containing bile salts (4.5 g/L) and pancreatin (1 g/L), and adjustment of the sample pH to 8.0, for an incubation period of 2 hours in an orbital shaker. Cell survival was assessed after each incubation of the dynamic model by enumeration on MRS agar. The same model was reported by Alves et al. [68] for testing free and microencapsulated cells, however, the simulation of gastric emptying was not described.

A completely automatized dynamic method was performed by Arroyo-López et al. [26], using the TNO gastro-Intestinal tract Model – TIM system (TNO-Triskelion, Zeist,

Netherlands). Moreover, the olive isolates were inoculated in a solution consisting of the homogenization of pasteurized olives in sterile water, working as food matrix. The TIM system consists of an alternative dynamic computer-controlled, multi-compartmental *in vitro* system that simulates the physiological processes occurring in the stomach, duodenum, jejunum, and ileum; it was accordingly programmed to reproduce the digestion of a solid food matrix by a healthy human adult, considering a total duration of digestion of 5 hours.

### 4.6.3. Critical assessment of analytical methods

The carrier in which the microorganisms were tested varied among the different methods. In most papers, the isolates were suspended in an eletrolyte solution or PBS modified so as to mimic the GI conditions – which theoretically lead to reliable results, since it does neither favour nor unfavour their response to the harsh conditions prevailing in the human GI tract. On the other hand, the use of pasteurized olives as food matrix in the assay allows a closer simulation of the *in vivo* conditions of the ingestion of microorganisms. More realistic conclusions may be derived on the ability of certain strains to keep their viability through the GI tract when carried in olives. However, there is not much information on the use of olives as food matrix for the assessment of survival of microorganisms across the GI tract; and complex systems may be needed to perform the simulation of digestion of a solid food matrix [26].

Since the ingestion of a healthy dose of table olives can carry $10^9$-$10^{10}$ CFU of selected *Lactobacillus* spp., controling the concentration of cells at the beginning of the assay, as accomplished in several studies, in order to start with a number of cells close to what is ingested, may lead a more significant comparison to *in vivo* digestion.

The majority of the *in vitro* methods to assess resistance to the passage on GI tract consisted of static bi-compartimental models comprising a gastric phase and an intestinal phase. Although several studies supplemented the SGj with lysozyme, which is typically found in saliva and known for its antimicrobial properties, they lack a first step of oral digestion - which could give valuable information on the capacity of their isolates to resist lysozyme. In addition, lysozyme is active over a broad range of pH (6-9), yet the conditions found in a gastric environment are too acidic and would thus hardly favour its action - as opposed to the conditions found in the mouth, where the pH of saliva is close to the optimal for the enzyme [73]. Therefore, supplementing the SGJ with lysozyme may not allow to evaluate the effect it would exhibit *in vivo*.

For a static model, where the conditions in each digestion batch are kept constant, the pH values, enzymes present and concentrations and periods of incubation for both gastric and intestinal digestion should be in the same range as analysed for the independent assays - and summarized in Figure 2.
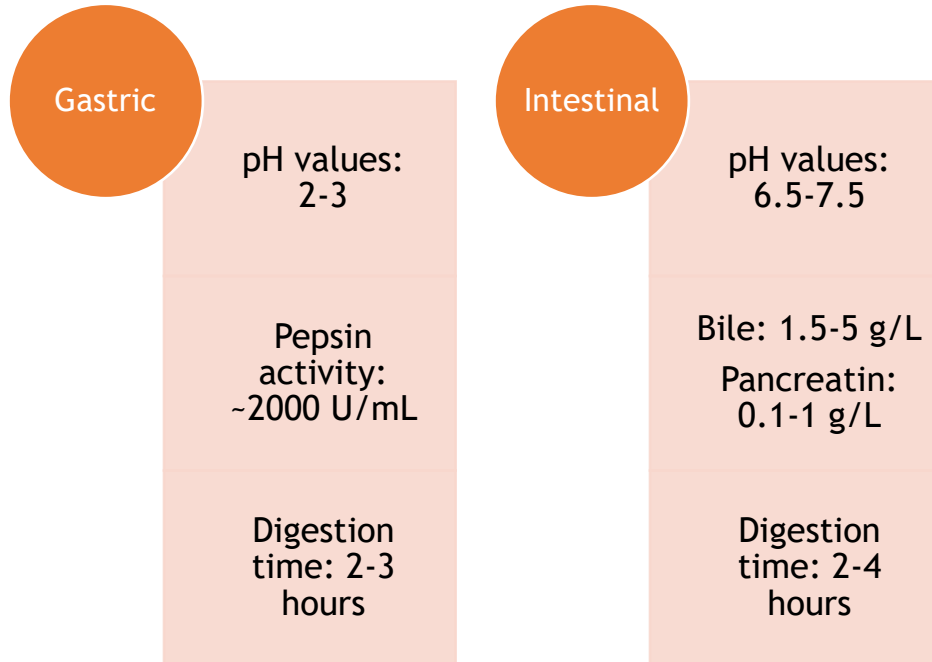
**Gastric**

pH values:
2-3

Pepsin
activity:
~2000 U/mL

Digestion
time: 2-3
hours

**Intestinal**

pH values:
6.5-7.5

Bile: 1.5-5 g/L
Pancreatin:
0.1-1 g/L

Digestion
time: 2-4
hours

**Figure 3.** Conditions recommended for a static simulation of GI digestion

Regarding the gastric digestion phase, most authors indeed performed the assays within the parameters previously suggested. However exposure to gastric conditions for merely 1 hour as done by Guantario et al. [66], may not assure that the isolates that survive the simulated gastric digestion and proceed viable to the pancreatic digestion, would in fact tolerate an *in vivo* digestion within the stomach and reach while viable to the small intestine. Moreover, inclusion of bile salts in the gastric phase, as done by Botta et al. [7], has not practical relevance, due to a hardly consensual presence of this substance in all individuals, and the low levels observed when present [76]. The presence of the main digestive enzyme, pepsin, was also evaluated in all studies, although using different concentrations. As previously described, the use of pepsin at 2000 U/mL in the final digestion mixture is recommended [76]. Regarding the intestinal phase of digestion, all papers simulated it at pH between 7.2 and 8; these values are closer to the pH found along the small intestine, although a pH value of 8 is already significantly higher than the experienced in the duodenum (6.2 – 7), where the conditions are harsher [75, 97]. The absence of bile in the SIJ used by Montoro et al. [80] may lead to predictions of higher tolerance and, consequently, higher survival level of the isolates than what would happen in real digestion within the intestine. Furthermore, trypsin is just one of the

many enzymes segregated in the small intestine; therefore the addition of pancreatin, which includes all the major pancreatic enzymes, is recommended for a reliable simulation of the enteric digestion [76]. The transit time through the whole small intestine goes around 3-4 hours, and a period between 2 and 4 hours is typically accepted when simulating it [98, 99]. Hence, checking survival after a much more extended period under pancreatic juice influence, as done by Montoro et al. [80], may underestimate the number of cells that maintain viability during GI transit.

Regarding the incorporation of nitrate in the GI simulation, as exclusively performed by Montoro et al. [39], it may not be important to assess the effect of this compound, even at low concentrations, on the isolates - since its content in fermented olives is almost vestigial, and probably does not accumulate to that level in the GI tract [102].

Indeed, due to its simplicity, static models that maintain a constant concentration of enzymes and other substances, and a constant pH for each digestion phase, have been widely used and have been shown quite useful in predicting outcomes of *in vivo* digestion [103, 104]. Despite the good predictability of static *in vitro* sequential digestion models, several factors are not taken into account - such as stomach emptying, or variations in pH and concentrations of bile or enzymes. They may accordingly cause some flaws in the predictions [95, 98]. For example, at the beginning of digestion, the pH in the stomach is entirely above the range of values usually tested in a static model. Many microbial cells may leave the organ as it empties while the pH is still relatively high and reaches the small intestine while viable, even if susceptible to acidic environments [92]. The method used by Peres et al. [67], based on a relatively simple dynamic model, considers the emptying of the stomach at increasingly acidic pH, and the consequent gradual passage of the food and the microorganisms from the stomach to the small intestine after each incubation – thus allowing simulation of the intestinal conditions on isolates that experienced different levels of pH on the gastric digestion, and consequently approaching what happens in reality. Therefore, this approach emphasizes the importance of the initial period of gastric emptying for the delivery of live bacteria into the small intestine; and its ability to predict the outcomes of *in vivo* digestion may be better than the static methods usually employed. However, the method considers a total duration of gastric digestion of 100 minutes, substantially below to the 3-4 hours of *in vivo* digestion. Moreover, as such assessment can be somewhat exhausting both in material utilized and time spent (due to extensive sampling across the simulation that makes it difficult to manipulate several strains at the same time), a more straightforward method may be required for pre-screening, to narrow the number of isolates to be tested. Indeed, Peres et al. [67] performed a pre-selection of the strains by inoculating them on

MRS agar plates adjusted to pH 3.5 or added with bile salts (3g/L), and checking the presence of growth, to assess acid or bile tolerance, respectively.

The TIM system used by Arroyo-López et al. [26] has greater complexity in its operation, with the passage of the food matrix through physical compartments functioning as the stomach and the different portions of small intestine and the simulation of the environment changes in each compartment of the GI tract during the different periods of digestion, through the constant flux of the main enzymes and chemical substances and the acidification of the stomach compartment as it empties. In fact, this in the only system that fulfil five important requirements when simulating the digestive system: sequential use of enzymes in physiological amounts; appropriate pH for the enzymes and addition of relevant cofactors such as bile salts and coenzymes; removal of the products of digestion; appropriate mixing at each stage of digestion; and physiological transit times for each step of digestion [31]. The application of such a process allows the closest simulation of *in vivo* physiological processes occurring within the stomach and small intestine of humans and, therefore, the selection of probiotic strains using these kind systems may be more reliable [32]. Nevertheless, the lack of a compartment or a step for simulation of the action of saliva prior to the gastric section could increase even more the similarities to the *in* vivo digestion. Despite its extremely reliable capability, the implementation of such a computed dynamic simulator is extremely complex and expensive; therefore, it is not within reach for the majority of laboratories [95].

### 4.6.4. Microorganisms tested

Again, the microorganisms isolated from table olives and further tested were lactic acid bacteria or yeasts (see Table 4).

**Table 4.** Types of microorganisms tested for resistance to sequential GI conditions

| Lactic acid bacteria | Yeast | Reference |
|:---:|:---:|:---:|
| + | | Benitez-Cabello et al. (2019)[60] |
| | + | Porru et al. (2018)[61] |
| | + | Bonatsou et al. (2018)[14] |
| + | | Guantario et al. (2018)[66] |
| | + | Oliveira et al. (2017)[62] |
| + | | Prete et al. (2017)[65] |
| | + | Montoro et al. (2016)[80] |
| + | | Alves et al. (2015)[68] |
| | + | Bonatsou et al. (2015)[63] |
| | + | Botta et al. (2014)[7] |
| + | | Arroyo-López et al. (2014)[26] |
| + | | Peres et al. (2014)[67] |
| + | | Bautista-Gallego et al. (2013)[64] |

Bacterial population tested belonged to the following species: *L. plantarum, L. pentosus, L. paraplantarum, L. coryniformis, L. oligofermentans Ln. mesenteroides, E. gallinarum* and *E. casseliflavus*.

Yeasts tested comprise members of the following genera and species: *Pichia guilliermondii, Pichia kluyveri, Pichia manshurica, P. membranaefaciens, C. silvae, Candida naeodendra, Candida diddensiae, C. boidinii, Candida molendinolei, C. norvegica, Candida tropicalis, C, matritensis, Cystofilobasidium bisporidii, M. pulcherrima, S. cerevisiae, Rhodotorula mucilaginosa, Rhodotorula diobovatum, Rhodotorula glutinis, Rhodotorula graminis, Aereobasidium pullulans, Debaryomyces*

*hansenii, Galactomyces reessii, Nakazawaea molendini-olei, Wickerhamomyces anomalus, Zygotorulaspora mrakii* and *Zygoascus hellenicus*.

# 5. Conclusions and Future Work

The present dissertation focused on the compilation and review of methodologies employed in studies from the past ten years to evaluate the resistance to the passage on the GI tract of microorganisms isolated from table olives – as long as it is considered a crucial feature for claiming a probiotic potential. Different approaches were followed when evaluating this property, since assessing the resistance to conditions simulating the environment within the stomach or the intestine, independently or by combining the two tests, or exposing the microorganisms to the two stresses successively, as happens when food is ingested and enters the GI tract. A critical analysis of the different methods used enables a rational selection of a protocol for studying resistance to digestion of isolates from table olives in a future practical work, thus supporting a higher probability to obtain reliable results. Furthermore, the comparison of the different methodologies may lead to some adjustments on the protocol selected, in order to make the conditions on the assay closer to an *in vivo* situation.

Many authors followed more straightforward methods, assessing independently the tolerance of isolates to gastric and intestinal conditions. In most cases, the resistance to low pH and the action of bile were the main features evaluated to assess the capability to survive within the gastric and intestinal tracts, respectively – although in some cases the influence of the main digestive enzymes (pepsin and pancreatin) was also included when simulating the digestive juices. However, during digestion, the food does not experience gastric and intestinal conditions independently from each other, but rather both stresses are immediately successive; hence, the possibility exists that an initial stress may influence the susceptibility of cells to further stresses. Therefore, the remaining authors performed *in vitro* methods of sequential digestion, where the cells harvested from exposure to oral or gastric digestion proceed to the next phase of the GI tract. The majority employed static *in vitro* sequential models to simulate passage through the GI tract, which allow adequate predictions of the microorganisms' behaviour on *in vivo* digestion. Even so, dynamic models that, depending on their complexity, can simulate the process of digestion in a way closer to reality (considering the dynamic that exists within each GI compartment) have also been employed. However, if the complexity of a model is too high, it may not be easily available to most laboratories.

From the critical analysis and comparison between the methods, it was possible to choose a method to perform in a future laboratory project. The dynamic model used by Peres et al. [67] exhibits advantages in relation to the static models reviewed, while being simple enough to be performed in a regular laboratory. Therefore, the protocol to be implemented in the future in the laboratory is based on the method executed by Peres

et al. [67], with some modifications, considering the critical discussion presented above. The whole process for assessing resistance to the passage through GI tract is represented in Figure 4. The lactobacilli will be propagated in MRS broth, overnight, in order to achieve stationary phase, harvested by centrifugation and re-suspended in PBS adjusted to pH 6.2, adjusted with hydrochloric acid, to a final concentration between $10^7$-$10^{10}$ CFU/mL – thus matching the quantities ingested within a normal dose of table olives; and an aliquot is withdrawn to serve as control (sample G1). Then, in order to simulate *in vivo* saliva, lysozyme is added to the solution to a final concentration of 0.1 g/L, and the suspension is incubated for 2 minutes at 37 °C, in an orbital shaker, before an aliquot is taken (sample G2). For the gastric simulation, the suspension is adjusted to pH 5.0, and supplemented with pepsin to a final concentration of 2000 U/mL. Then, the pH curve in the stomach is reproduced by adding 1 M HCl to the cell suspension, at pH 5 that is gradually decreased to 4.1, 3.0, 2.1 and 1.8. The stomach emptying is simulated by collecting fractions of the suspension after sequential incubations of 30 min, at 37 °C in an orbital shaker, at each pH value (samples G3-G7), for a total gastric digestion duration of 2.5 hours. In the simulation of the intestinal phase of digestion, samples G3-G5 are adjusted to pH 6.5 with 1 M NaHCO₃, added with bile salts (5 g/L) and pancreatin (1 g/L), and then adjusted to pH 7.0. After a period of incubation of 2 hours, aliquots from the different samples are taken (GI3, GI4 and GI5). The survival assessment in each stage of the dynamic digestion is performed by pour-plating in MRS agar.
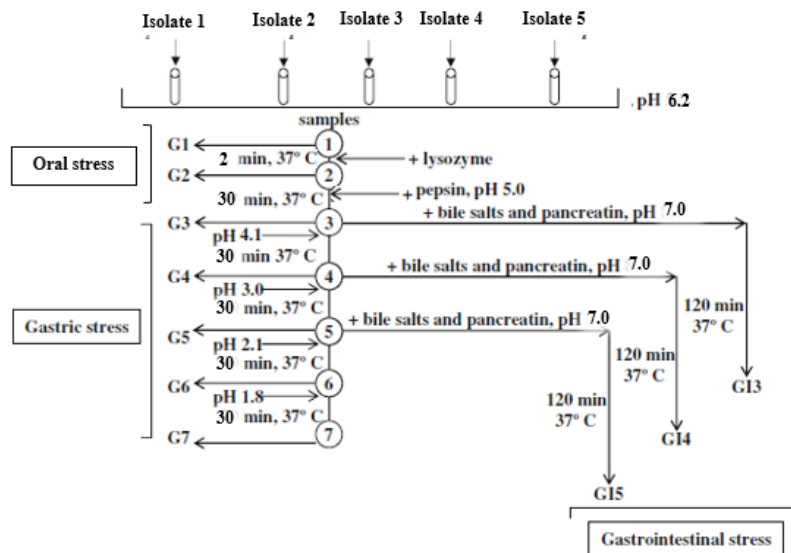


**Figure 4.** Schematic representation of the *in vitro* digestive tract model. Adapted from Palencia et al. [101]

# 6. Limitations

The occurrence of a pandemic caused by the COVID-19 virus imposed major restrictions in the fulfilment of this Master Thesis as per its original plan. The possibility of contagious contact and the state of emergency enacted in the country caused the university facilities to close and, consequently, compromised a project based on practical laboratory work. Therefore, there was a need to come with an alternative project based on a theoretical study to replace the original focus of project.

Concerning the work done with respect to the review, it was difficult to present a final unique protocol - considering the variability among methods; and for those similar, the variability among parameters (such as pH, temperature, and time).

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