Escuela Agrícola Panamericana, Zamorano

Food Science and Technology Department

Food Science and Technology Major

Graduation Research Project

Exploring the Impact of Novel Prebiotic Substrates on *Lactobacillus casei* and *Lactobacillus acidophilus* under bile stress exposure

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Honduras, november 2023

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Abstract

The increase of interest in probiotics to promote better health shows a substantial promise to expand the food industry into new fields. The gut microbiota has several beneficial effects on human health and the search for enhancing it to maintain and foster a larger amount of these probiotics has led to the emergence of probiotics, prebiotics, and symbiotics products. It has been identified that Carao (Cassia grandis), Teosinte (*Dioon mejiae*), South American palm weevil (*Rhynchophorus palmarum*), and Caesar's mushroom (*Amanita cesarea*) have bioactive compounds with prebiotic characteristics and significant nutritional applications. The study aims to carry out a literary review of probiotics and prebiotics and their relationship with the human microbiome, and also to investigate the behavior of *L. casei* and *L. acidophilus* under bile (Oxgall 0.3%) stress in the presence of Carao, Teosinte, ceasar mushroom and South American palm weevil. The software Statistical Analysis Systems (SAS) was used and examined using the General Linear Model (PROC GLM) for the main effects (treatments and time). Duncan test was used to determine the statistical differences ($p < 0.05$) among the main effects. In the case of the literary review, 64 documents were reviewed, of which 11 did not meet the established criteria. The selected ones cover the microbiome, probiotics, their relationship in the digestive tract and bile tolerance evaluation. The analyzes showed that for both bacteria *L. acidophilus* and *L. casei*, treatments with prebiotic potential did not reduce biliary stress; in addition, the biliary stress generated (0.3%) despite being a similar concentration of small intestine this concentration acts as sublethal stress to *L. casei* and *L. acidophilus*.

Keywords: Gut microbiota, probiotics, symbiotics products.

Resumen

El incremento del interés en los probióticos para promover una mejor salud muestra una promesa sustancial para expandir la industria alimentaria hacia nuevos campos. La microbiota intestinal tiene varios efectos beneficiosos en la salud humana y la búsqueda por potenciarla para mantener y fomentar una mayor cantidad de estos probióticos ha llevado al surgimiento de productos probióticos, prebióticos y simbióticos. Se ha identificado que el Carao (*Cassia grandis*), el Teosinte (*Dioon mejiae*), el gorgojo sudamericano de la palma (*Rhynchophorus palmarum*) y la seta de César (Amanita cesarea) poseen compuestos bioactivos con características prebióticas y aplicaciones nutricionales significativas. El estudio tiene como objetivos llevar a cabo una revisión de literatura de probióticos y prebióticos y su relación con la microbiota humana. También investigar el comportamiento de *L. casei* y *L. acidophilus* bajo estrés biliar (Oxgall 0.3%) en presencia de Carao, teosinte, seta de César y gorgojo sudamericano de la palma. Se utilizó el software Sistema de Análisis Estadístico (SAS) y se examinó mediante el Modelo Lineal General (PROC GLM) para los efectos principales (tratamientos y tiempo). La prueba de Duncan se utilizó para determinar las diferencias estadísticas ($p < 0.05$) entre los efectos principales. Los análisis mostraron que, para ambas bacterias, *L. acidophilus* y *L. casei*, los tratamientos con potencial prebiótico no redujeron el estrés biliar; además, el estrés biliar generado (0.3%), a pesar de ser una concentración similar al intestino delgado, actúa como estrés subletal para *L. casei* y *L. acidophilus*. En el caso de la revisión literaria, se revisaron 64 documentos, de los cuales 11 no cumplieron con los criterios establecidos. Los seleccionados abarcan el microbioma, los probióticos, su relación en el tracto digestivo y la evaluación de la tolerancia biliar.

Palabras clave: Microbiota intestinal, probióticos, productos simbióticos.

Introduction

The recent increase in awareness towards human nutrition, especially in the developed countries, has brought the search for optimal nutrition. This is evident in the rapid growth of interest in probiotics to promote better health and well-being which shows a substantial promise to expand the food industry into new fields. The gut microbiota has several beneficial effects on human health, including promoting innate and adaptive immunity, maintaining the intestinal epithelial integrity, helping the metabolism and synthesis essential of nutrients such as vitamins, carbohydrate, and resisting pathogens (Yin et al., 2019).

Probiotics were defined in 2002 by FAO (Food and Agriculture Organization of the United Nations) and WHO (World Health Organization) working group experts, as “live strains of strictly selected microorganisms which, when administered in adequate amounts, confer a health benefit on the host”. Probiotics are dietary factors that can influence the human gut microbiota and have a regulatory effect on the composition and structure of the intestinal flora (Xinzhou Wang et al., 2021). However, probiotic microorganisms must meet some criteria to ensure their delivery in sufficient amounts in their action site so they could exert beneficial effects, then, they must undergo and survive under very harsh conditions before reaching the lower intestinal tract. After oral administration, probiotics pass through the gastrointestinal tract (GIT), from the mouth, through the stomach, to the small intestine and colon (Han et al., 2021). Bile acids and salts, as well as digestive enzymes (including lipases, proteases, and amylases) present in the small intestine can impact probiotic viability through cell membrane disruption and DNA damage. Bile salt concentration generally varies from 0.2–2.0% in the small intestine, although dietary intake and nourishment status can greatly affect bile levels (Prouty et al., 2004). Probiotic properties, functionality and benefits differ among strains. Lactic acid bacteria (LAB), such as *Lactobacillus*, are the most relevant and consumed probiotics in the food industry.

L. acidophilus has been described to have good probiotic characteristics in terms of acid tolerance, bile tolerance, antibiotic sensitivity and antibacterial activity against different pathogens and could be used as potential functional probiotics in food industry for commercial use (Sharoba et al., 2015). These characteristics facilitate the survival and proliferation of *L. acidophilus* in the harsh environment of the gastrointestinal tract. According to Aryana and Olson (2017) *L. casei* has been extensively studied as a fermentation starter culture and as a probiotic and exhibited intensively probiotic properties such as strong survival in acidic pH conditions, bile tolerance (Divyashree et al., 2021). Viable probiotics that manage to reach the colon must also compete with the host microbiota for nutrients and adhesion sites to be able to colonize the colonic mucosa and proliferate (Zmora et al., 2018), hence the emergence of prebiotics as a strategy to help probiotics colonize and proliferate in the colon.

In 2007, FAO/WHO experts described prebiotics as a nonviable food component that confers a health benefit on the host associated with modulation of the microbiota. Prebiotics have enormous potential for modifying the gut microbiota, but these modifications occur at the level of individual strains and species and are not easily predicted in advance. Fruit, vegetables, cereals, and other edible plants are sources of carbohydrates constituting potential prebiotics (Markowiak & Śliżewska, 2017). Prebiotics may be used as an alternative to probiotics or as an additional support for them. Prebiotic substances selectively stimulate microorganisms present in the host's intestinal ecosystem, thus eliminating the need for competition with bacteria. Additionally, resistance to acids, proteases, and bile salts present in the gastrointestinal tract may be considered as other favorable properties of prebiotics (Markowiak & Śliżewska, 2017). Because a probiotic is essentially active in the small and large intestine, and the effect of a prebiotic is observed mainly in the large intestine, the combination of the two may have a synergistic effect (Hamasalim, 2016; Markowiak & Śliżewska, 2017). Symbiotics have both probiotic and prebiotic properties and were created to overcome some difficulties in the survival of probiotics in the gastrointestinal tract. For the aim of the study

substrates Carao (*Cassia grandis*), Teosinte (*Dioon mejiae),* Ceasar mushroom (*Amanita caesarea),* South American palm weevil *(Rhynchophorus palmarum)* were used as potential prebiotics substrates.

Cassia grandis (carao), have shown considerable amounts of alkaloids, flavonoids, and phenols, and excellent antioxidant capacity (Kotipalli Harika et al., 2017) and has been shown to improve acid and bile tolerance of Streptococcus thermophilus and Lactobacillus bulgaricus (Paz et al., 2022). The South American palm weevil*, Rhynchophorus palmarum*, is a vital source of protein and has been consumed by Indigenous populations, and has been linked to have a good antioxidant activity (Aleman et al., 2023). Teosinte, a wild relative of maize sourced from *Dioon mejiae*, has been described as a highly caloric food with high total carbohydrate content that mainly consists of starch (Bastias-Montes et al., 2020). Lastly, Ceasar mushroom, identified as *Amanita caesarea*, has been hinted at its antioxidant and anti-inflammatory potential (Doğan & Akbaş, 2013) and is an excellent source of protein, fiber, and minerals (Aleman et al., 2023).

In this context, this study aims to carry out a literary review of probiotics and prebiotics and their relationship with the human microbiome. Also, aims to investigate the behavior *L. casei* and *L. acidophilus* under bile stress (Oxgall 0.3%) conditions in the presence of carao, Teosinte, Ceasar mushroom and South American palm weevil.

Materials and Methods

Phase 1. Literature Review

Study Location

The research was carried out at the Panamericana Zamorano Agricultural School, km 30 highway from Tegucigalpa to Danlí, Valle del Yeguare, Municipality of San Antonio de Oriente, Francisco Morazán, Republic of Honduras.

Design of the Investigation

An exhaustive review of the documentation related to probiotic microorganisms and their effects on the health of consumers was conducted. The search was carried out using primary and secondary sources of information related to the topic described above, the review was descriptive in nature, understood as an initial approach to a changing topic, the breadth of the topic was addressed through the research design, strategy search, and application of exclusion and inclusion criteria, to structure and rationalize the concepts applied in the literature review.

Research Strategy

The review was conducted using scientific databases available on the web such as PubMed, Scopus, Web of Science, Springer, SciELO, and eLIBRO. For the documentation used, the age of publication was restricted to 7 years, The review was conducted on documentation with direct or indirect linkage on probiotic microorganisms, prebiotic materials, or their relationship, all the information was related with human microbiota, to include information that consolidates the subject.

Criteria for Inclusion and Exclusion

The search focused on scientific publications such as articles and studies whose central focus is probiotics as well as those that address human-food-probiotic interactions. In the topic, the basic exclusion criterion for the information deposited in the databases consulted was the presence of

keywords such as probiotics, intestinal health, division of labor, clinical applications of pro and prebiotics, among others. Scientific publication years had a maximum of 7 years (2016-2023) and was set as an exclusion criterion for the review.

Data Extraction

The information was preliminarily selected based on an exploratory review for each primary source, to then delve deeper into aspects of interest in the documentation. The search was also expanded with references cited in the primary source. This allowed us to extract information according to the proposed topic and that together formed the objective. The structure generated from the literature review was about intestinal microbiomes, probiotics, prebiotics, symbiotic, food-human-probiotic interaction.

Phase 2. Experimentation on New Prebiotic Substrates in *Lactobacillus casei* and *Lactobacillus acidophilus*

Location

The study was carried out in the facilities of Louisiana State University, located in the city of Baton Rouge, Louisiana. The tests on the microorganisms of interest were performed in the dairy microbiology laboratory.

Substrates Used

Carao (*Cassia Grandis*), Caesar's mushrooms (*Amanita caesarea*), Teosinte (*Dioon majiae*), and Weevils (*Rhynchophorus palmarun*) were the four substrates with tentative probiotic potential on microorganisms used for this study.

For obtaining and handling of biological material the *C. grandis* (Carao) fruit was gathered from the Guapinol Biological Reserve, Marcovia Municipality, Choluteca Department (Honduras). The pulp was separated in a solution of Carao pulp (10% w/w) and kept cryogenically (−80 °C). The obtained Carao aqueous solution was then lyophilized (LIOTOP model L 101) for 48 h at a

temperature of −75 °C and a chamber pressure of 0.1 to 0.5 Pa. The freeze-dried Carao pulp powder was kept in plastic bags for further use.

Caesar's mushrooms were collected from the Guapinol Biological Reserve, Marcovia Municipality, Choluteca Department (Honduras). Teosinte was collected from Rio Grande, Saguay, Olancho. Teosinte (*Dioon mejiae)* and Caesar's mushroom (*Amanita caesarea)* were individually placed in water solution (10% *w*/*w*), and then held at −80 °C temperature to freeze-dry in an Labconco (model L 101, Kansas City, MO, USA) lyophilizer, for 48 h, at −75 °C and 0.5 Pa. After lyophilization, the extracts were ground and stored in vacuum-pack plastic bags for further use.

Adult weevils were obtained from the entomology laboratory at the National University of Agriculture (UNAG, Honduras). Weevils were dehydrated in a convection oven (Digitronic TFT-Selecta, J.P. SELECTA, Barcelona, Spain) at 50 °C/48 h, and were grounded using a knife mill Retsch SM 100 (Retsch GmbH, Haan, Germany) (501–700 mm) [Aleman, R. et al. 2023]

Strains Used

L. casei and *L. acidophilus* strains (Chr. Hansen, Milwaukee, WI) due to their probiotic characteristics were selected. Strains were stored in Laboratory Freezer (LABRepCo) at -40 °C. Before analysis, strains were activated. *L. casei* was passaged in MRS broth (de man, Rogosa, Sharpe) and incubated at 37 °C for 24 hours under anaerobic conditions. *L. acidophilus* was passaged in MRS broth (de man, Rogosa Sharpe) at and 43 °C for 24 hours, anaerobically.

Broth Preparation

L. casei and *L. acidophilus* were evaluated for their ability to grow in MRS broth (Criterion™, Hardy Diagnostics, Santa Maria, CA). Each evaluation had four substrates of testing at 2%: Carao, teosinte, South American Weevil, and Ceasar Mushroom. Broths with the addition of the substrates were prepared, and then adjusted to simulate a stressful environment (specifications ahead). Broths were prepared before inoculation of the two probiotic strains.

The substrates with probiotic potential are shown in Table 1, as well as the resulting composition of media with the addition of the substrates.

Table 1

Prebiotic treatments and culture media composition

Treatments	Description
TRT 1	MRS broth + Carao (2%)
TRT 2	MRS broth + Teosinte (2%)
TRT 3	MRS broth + C. mushroom (2%)
TRT 4	MRS broth + Weevil (2%)
Control	MRS broth

Note. MRS means deMan-Rogosa-Sharpe. Prebiotics are at 2% of total culture media composition.

Stress Condition and Inoculation

Bile tolerance of *L. casei* and *L. acidophilus* was analyzed according Liong and Shah (2005) with slight modifications. Five testing tubes were prepared using MRS broth with 0.3% of Oxgall (bovine bile), in four of the tubes Carao, Teosinte, C. mushroom, weevil was added at 2%, and one tube was absent of any substrate (control). Broths were than inoculated using 5 mL of each probiotic culture, which represents a concentration of 11.6 log CFU.

For bile tolerance one milliliter sample of each serially 8-fold diluted peptone water was taken at various times (0, 4, and 8 hours), simulating the passage through the small intestine were bile salts are present, and plated onto MRS agar (Difco, Detroit, MI).

Enumeration of Lactobacillus casei and Lactobacillus acidophilus

Serial-8 dilutions were prepared from all 5 broth inoculated with *L. casei* and *L. acidohpilus* and adjusted to bile stress. Dilutions were than plated using the pouring plate tecnique using MRS agar (prepared as manufacture instructs). Samples were than incubated incubated anaerobically at 37 ºC and 43 ºC for 72 h. To enumerate the colonies a Quebec Darkfield Colony Counter (Leica Inc., Buffalo, NY) was used.

Experimental Design and Statistical Analysis

L. acidophilus and *L. casei* were evaluated in the presence of four different subtracts at 2% and a control (absence of substrates) under bile stress, with samplings taken in three periods of time over 8 hours (0, 4, 8 hours). The experiment was done on triplicate, which represents ninety experimental units.

The data were analyzed using the General Linear Model (PROC GLM) for the main effects (substrates and time). A completely randomized design was used with measurements over time. Separation of means was performed with Duncan's test to determine statistical differences ($p < 0.05$) between the main effects (treatments and time) at $\alpha = 0.05$. The data were processed in Statistical Analysis Systems (SAS). SAS version 9.4 was used.

Results and Discussions

Phase 1. Literature Review

Gut Microbiota

Humans, like other multicellular organisms, have a complex system of interactions with microorganisms present in various parts of the body, one of the most important being the intestinal microbiota (Pascual et al., 2022), which is defined as a complex and dynamic population of microorganisms that develop in the human digestive tract (Thursby & Juge, 2017). The diversity and interactions of the populations that make up the intestinal microbiome have significant effects on not only gastrointestinal but also immunological and neurological health, due to this the interest in human microbiomes has increased considerably in recent years (Shi et al., 2017). Although the functions of the microbiota are varied, we can mention the prevention of colonization of the gastrointestinal system by pathogens, stimulating the immune system and the production of compounds that cannot be synthesized by humans (Del Campo-Moreno et al., 2018).

Beneficial Microorganisms

They are defined as microorganisms that have beneficial effects on the gastrointestinal health of humans (Panthee et al., 2022). Such effects are explained by the interactions it has on the other populations present in the intestinal microbiome, mainly in the promotion of beneficial populations in decline or in the interference in potential pathogenic populations (Sánchez et al., 2017). The use of probiotics addresses the need to increase the amount of these beneficial organisms in the intestinal flora to promote positive effects on human health (Salvucci, 2019).

Potentially Pathogenic Microorganisms

In the human microbiome there are two types of pathogens, the exogenous ones that come from outside the organism, which enter through the ingestion of contaminated food and the so-called autochthonous pathobionts (Rolhion & Chassaing, 2016), defined as microorganisms native to the human microbiome that are potential pathogens when their number increases, affecting the

population balance of microbial communities (Buret et al., 2019). The human microbiome has developed a process of regulating pathobionts using other species to keep potentially pathogenic populations at bay and in the case of exogenous pathogens there is resistance to colonization (Bäumler & Sperandio, 2016).

Microorganisms in Equilibrium

Human microbiomes are highly resilient ecological systems, in the case of any abrupt change in the composition of microbial communities, such as for example the intensive application of antibiotics, the system seeks to return to its initial level in terms of diversity and composition of microorganisms (Greenhalgh et al., 2016), the balance of microorganisms is a crucial state in the homeostatic process where the populations of a healthy microbiome remains globally stable throughout the life of the human (Venturelli et al., 2018), in this condition the microorganisms that compose the human microbial system are in a condition of equilibrium in the various populations that compose it, in other words one population does not predominate over another (Das & Nair, 2019).

Probiotics

They are live microorganisms that colonize the human digestive tract in its last segments, understanding the large intestine as the main place of residence (Plaza-Diaz et al., 2019)siv , colonization effect these microorganisms can adjust the microbial communities present having significant effects on human health by inhibiting pathogenic organisms and collaborating in the construction of protective mucosal layers in the large intestine (You et al., 2022), the mechanisms of action played by probiotics in addition to the interactions with the other populations of the microbiome are currently under extensive study due to the interest in the relationship of the microbiome with various human diseases (Bozzetti & Senger, 2022).

Probiotics Characteristics

Although the mechanisms of action exerted by probiotics on the communities that make up the human microbiome still have some degree of uncertainty (Monteagudo-Mera et al., 2019), competitive exclusion, in which probiotics reduce the growth of pathogens or potential pathogens by occupying large regions of the large intestine (Latif et al., 2023), another mechanism identified is the improvement in the functions of the intestinal barrier, immunomodulation in the gastrointestinal tract and in the production of neurotransmitters (Plaza-Diaz et al., 2019), the aforementioned are the main mechanisms identified in probiotics that favor human health, it can be synthesized that probiotics regulate the populations present in the human microbiome.

Prebiotics

Prebiotics are food groups that contain high levels of nondigestible components, commonly fiber, this material is colonized by microorganisms of the gastrointestinal tract, being degraded into its constituents, which are used by the intestinal microbiome promoting the growth of beneficial microorganisms for the organism. The degradation of prebiotics are mostly short-chain grade acids that are released into the bloodstream by intestinal absorption this makes prebiotics not only modify intestinal health but also has direct effects on distant organs (Davani-Davari et al., 2019), recently with advances in community genomics shows that the interactions developed between probiotics and the intestinal microbiota is even more complex than estimated in previous studies (Hutkins et al., 2016).

Symbiotics

The term refers to products that contain prebiotic and probiotic components, specifically products that have prebiotic components that selectively favor the growth of the probiotic microorganism it contains, generally the symbiotic components is a compound or family of specific compounds consider the case of polysaccharides or oligosaccharides with an isolated strain of microorganism (Olveira & González-Molero, 2016).

Lactobacillus acidophillus

Lactic acid bacteria (LAB) belonging to the genus Lactobacillus of the Gram-positive type, especially studied for the attribution of probiotic properties, currently used in a multitude of processes including the processing of fermented dairy products as well as other fermented products of the food industry (Peng et al., 2021). This microorganism has been found to be an important probiotic closely related to human health, and it is one of the most recommended microorganisms for dietary purposes. Among the characteristics of this LAB is high resistance to acid and bile salts facilitating its survival and proliferation in the gastrointestinal tract where the above conditions prevail (Gao et al., 2022).

Lactobacillus casei

Lactobacillus casei is among some of the most studied species due to their commercial, industrial, and applied health potential. Commercially, they are used to ferment dairy products, often producing foods with improved flavor and texture. Health benefits associated with the strain have been reported for a variety of health conditions, ranging from atopic dermatitis to cancer (Hill et al., 2018). The mechanisms by which these bacteria directly or indirectly have a beneficial effect on human health are not yet fully understood and require further study, potential mechanisms include the production of antimicrobial substances such as bacteriocins, enhancing the epithelial barrier through attachment, competition for pathogenic binding sites, or modulation of the immune system (Bermudez-Brito et al., 2012; Hill et al., 2018). As probiotics the *L. casei* must endure many stresses during processing and remain viable on transit to their site of action, the gastrointestinal tract. These stresses include but are not limited to bile salts, oxidative stress, cold stress, osmotic stress, acid stress and long term storage (Hill et al., 2018).

Probiotic-gastrointestinal Tract Relationship

Microorganisms are found throughout the digestive system from the gastric juice with a few thousand bacteria per milliliter to the colon where concentrations of the order of 1011 to 1012 bacteria per ml are reached, the latter being the main reserve with 90% of the microbiota (Parizadeh

& Arrieta, 2023), in the case of probiotics they travel through the gastric system. When they are ingested, they are exposed to salvation, which is a secretion with immunological components (Johnson et al., 2016), then the probiotics reach the stomach where they are affected by gastric juice of an acidic nature, this condition being lethal for most of the microorganisms, upon arrival in the small intestine the pH conditions are neutralized, however, the microorganisms are exposed to pancreatic juices and bile salts, substances that reduce cell viability (Yao et al., 2020). This step is widely investigated under bile tolerance methodologies, finally the probiotics reach the large intestine specifically the colon, where the probiotics will begin colonization (Zmora et al., 2018), this process is opposed by the microbiota through competition which results in fecal expulsion of the probiotics (Gilbert et al., 2018).

As probiotics pass through the digestive tract they pass through points called critical points, where cell viability is considerably reduced, where they are mainly the stomach with an extremely acidic environment, the small intestine with bile salts and the colon with the phenomenon of resistance to colonization (Han et al., 2021).

Duodenum

The duodenum is the part of the small intestine where the bile ducts discharge bile salts. This segment of the small intestine is important for the application of prebiotics due to the reduction in cell viability affecting the host's microbiome (Lee et al., 2022), this is reflected in a scarce microbiota as a result of the toxicity of bile and the enzymatic degradation of pancreatic juice (Usca-Méndez et al., 2020), which is the importance of the duodenum in the in vitro simulation of food digestion with probiotics, which allows us to understand the effect of intestinal components on ingested microbial populations (Bamigbade et al., 2022).

Bile

The primary bile acids (BAs) are synthetized from cholesterol in the liver, conjugated to glycine or taurine to increase their solubility, secreted into bile, concentrated in the gallbladder

during fasting, and expelled in the intestine in response to dietary fat (Di Ciaula et al., 2017). Bile acids (sometimes referred to as salts) are secreted into the intestine of mammals to aid digestion (Hill et al., 2018) and offer another hurdle for bacteria as they have antimicrobial activity. Bile is known to affect the structure of membranes, DNA, RNA, and protein folding.

Bile Stress

Biliary stress is the damaged result that bile salts have on the bacterial cell, the presence of bile salts in growth media is more harmful than low pH, due to the alterations generated in the cell membrane of the probiotics (Ilango et al., 2016), the ability to resist biliary stress is called bile tolerance, a capacity dependent on the strain used and its ability to produce the enzyme hydrolase (Li et al., 2021), the ability to tolerate bile stress of a strain is vital in a probiotic because it defines when it will reach the large intestine where 10^8 CFU/G is required (Fiore et al., 2020).

Bile Salts

Bile is a liquid produced by the gallbladder that is involved in the emulsification of lipids ingested in food. Water predominates in its composition, but the active agent is bile salts, which are sodium or potassium salts with emulsifying power (Hundt et al., 2022), the composition of bile is divided into three important lipids, bile ducts, phosphatidylcholine and cholesterol, which together generate mixed micelles that allow the digestion and absorption of fats (Gómez, 2019).

Bile Salts on Probiotics

The effect of bile salts on probiotics occurs at the level of the cytoplasmic membrane. These salts alter proteins, which leads to the formation of defective proteins. Salts can also be introduced directly into the lipids that form the bilayer of the membrane. cellular, generating structural instability and permeability (Sivamaruthi et al., 2020). Another interaction between probiotics and bile salts is the production of free radicals by the salts that subsequently damage the genetic material of the microbial cells, reducing their viability (Gadaleta et al., 2022).

Methodologies for the Evaluation of Bile Tolerance in Probiotics

Currently there is no consensus on a standardized test method for assessing the stress imposed on a probiotic. Typically, a strain of the microorganism of interest is used, which is exposed to the stress factor, such as bile, to determine its tolerance (Wendel, 2021). The procedure, although varying considerably, can include fundamental steps in the evaluation of tolerance to bile in probiotics, as proposed by (Junnarkar et al., 2018) This includes: selection of the strain, application of the microorganisms in MRS broth with a concentration of 0.3% bile, and incubation of the medium at 37°C for 24 hours. This differs from other studies, such as that conducted by Lin et al. (2020) which used a concentration of 1.1% bile. Generally, the percentage of bile added ranges between the values found in the human duodenum, from 0.05% to 3% (Ayyash et al., 2021).

Pouring Plate Technique

This technique is commonly used in tolerance studies for probiotics, this method is often useful for counting the number of probiotics in a mixed sample, resulting in colonies with a uniform distribution in the medium, serial dilutions are used to complement the technique (Terrones-Fernandez et al., 2023). The technique begins by diluting the sample to the desired concentration of probiotics, then depositing a subsample on Petri dishes where the culture medium will be poured (Tovar Oviedo & Martínez Gutiérrez, 2017).

MRS Agar

The De man-Rogosa-Sharpe agar medium (MRS agar) is a medium widely used in the evaluation of tolerance of probiotics, specifically those belonging to the genus Lactobacillus, being a selective medium (Gnanasekaran et al., 2020) the MRS medium for testing of tolerance can be used in broth and agar under the split plate technique, incubating at 37 °C for 48 hours (Śliżewska & Chlebicz-Wójcik, 2020).

Oxgall

It is a commercially isolated form of bile from ox that is widely used in the analysis of bile tolerances in microorganisms from the gastrointestinal tract. Its similarity to human bile in terms of

composition allows the emulation of the conditions of the duodenum for the evaluation of potential microorganisms (Hu et al., 2018).

Phase 2. Bile Tolerance Test

Tolerance to bile salts is an essential capacity in probiotic organisms, since it determines the survival of probiotics in the digestive tract, tolerance to bile is multifactorial in nature (Ruiz et al., 2013), considering this affirmation, Table 2 shows the results of growth of *L. acidophillus* under bile stress using various substrates with prebiotic potential.

The behavior exhibited has two crucial points of interest: growth over time and the variation found among the prebiotic potentials, with respect to the first one, the treatments, except for time, did not present statistically significant differences, demonstrating that when compared with the control, none of the substrates with prebiotic potential had a positive impact on the performance of *L. acidophilus* under bile stress.

Table 2

Growth of L. acidophilus under bile stress after incorporating different treatments with sampling points over 8 hours

Treatments	Hour 0	Hour 4	Hour 8
	Log CFU/mL ± S.D.	Log CFU/mL ± S.D. ^{NS}	Log CFU/mL ± S.D. ^{NS}
Carao NS*	10.92 ± 0.29^{b}	10.47 ± 0.59	10.89 ± 0.31
Teosinte NS*	11.15 ± 0.52^{a}	11.11 ± 0.41	10.94 ± 0.19
C. mushroom NS*	10.41 ± 0.18^{b}	10.54 ± 0.63	10.86 ± 0.34
Weevil NS*	11.21 ± 0.54^{a}	10.94 ± 0.74	10.89 ± 0.39
Control NS*	10.55 ± 0.41^{b}	10.29 ± 0.52	10.57 ± 0.10
P-value	0.02	0.53	0.09
C.V.	2.26	6.03	1.41

Note. S.D.: Standard Deviation; C.V.: Coefficient of variation; NS: represents no significant difference (P>0.05) between means of the treatments along the same column; NS*: represents no significant difference (P>0.05) between means of the hours evaluated for each treatment along the same row; Control represents the MRS agar without any potential prebiotic; ^{ab}Means with different lowercase letters for Treatment log CFU/mL indicate significance difference (P>0.05) in each column.

Contrary to what is known about *L. acidophilus* about the growth it presents and what was reported by Calderón Vargas (2017) who demonstrated with kinetic modeling, exponential increases in populations over time, Kim et al. (2001) also demonstrated considerable growth for the

microorganism starting 2 hours after inoculation of the media used. The non-significant growth in the research could be associated with the biliary stress factor, generated by the addition of bovine bile at a concentration of 0.3% to the culture media, which could affect the multiplication of the bacteria. Considering that the lethal and sublethal levels for *L. acidophilus* are 0.5% and 0.05% respectively (Kim et al., 2001), and that the concentration used in the bile study was 0.3%, it could be explaining the minor growth in the bacteria without being lethal, despite being a high concentration that resembles what happens in the human body.

So although the concentration was considerably higher than the reported sublethal doses of 0.05%, the behavior of no growth is congruent with that reported for sublethal doses that cause alterations in the functionality given physical or chemical factors that do not kill the microorganism but retard growth (Pérez Esteve & Rivas Soler, 2022).

With respect to the treatments used, Table 2 shows, except for the results at time 0, that there was no difference in the potential prebiotics evaluated. At time 0, it was observed that the control with the teosinte and palm weevil treatments had significant differences with respect to the other treatments, however, this behavior was not maintained over time, so an effect of treatments on *L. acidophilus.*

If we look at times four and eight hours, the treatments show that statistically they are equal to the control, indicating that the effect of the prebiotic potentials is reduced, this can be associated to what is stated by Xiao Wang et al. (2015), establishing functional prebiotics as generally oligosaccharides, polysaccharides, hydrolyzed proteins, or short-chain acids.

The treatments evaluated have complex high molecular weight molecules. If this and what was mentioned above about the relative simplicity of prebiotics is considered, these do not meet the characteristics of being prebiotics. In the case of beetles and fungi, they have non-native protein that can be used by *L. acidophilus*. Treatments with plant materials have the presence of oligosaccharides which are reported as potential prebiotics, but this behavior was not shown in the experiment, and it

is likely that between at stake is the part used of the plant in its prebiotic capacity due to its composition, the Carao bark being the one used in the experiment, whose composition in the trees is up to 40% lignin (Neiva et al., 2020), considering as reported by Díaz Gutiérrez (2018) that materials rich in lignin require hydrolysis to obtain prebiotic substrates, for teosinte the reason is equivalent, when using seeds with a high percentage of complex molecules such as lignin.

The other microorganism evaluated was *L. casei* whose results of growth under bile stress with different prebiotic potentials are shown in Table 3. Congruent to the previous microorganism, there are two focuses of interest in the composition of the experiment, the first is the growth of the treatment over time and the variation between treatments for a specific time.

Table 3

Growth of L. casei under bile stress after incorporating different treatments with sampling points at different periods of time over 8 hours

Treatments	Hour 0	Hour 4	Hour 8
	Log CFU/mL ± S.D.	Log CFU/mL ± S.D.	Log CFU/mL ± S.D. ^{NS}
Carao	7.57 ± 2.06 ^{ax}	09.05 ± 2.21 ^{abx}	9.36 ± 0.96 ^{ax}
Teosinte	10.75 ± 0.20 ^{ax}	10.23 ± 0.66 ^{ax}	10.81 ± 0.37 ^{ax}
C. mushroom	2.65 ± 0.94 ^{by}	2.30 ± 0.99 ^{by}	10.56 ± 0.19 ^{ax}
Weevil	8.24 ± 2.10 ^{ax}	9.64 ± 2.05 ^{ax}	10.10 ± 1.52 ^{ax}
Control	7.83 ± 1.48 ^{ax}	9.72 ± 0.57 ^{ax}	10.66 ± 0.56 ^{ax}
p-value	0.01	0.006	0.25
C.V.	22.26	16.75	7.4

Note. NS: No significance (P>0.05); S.D.: Standard Deviation; C.V.: Coefficient of variation; $^{a-b}$ Means within a same column along same test with different letter differ statistically ($p < 0.05$); $^{x-y}$ Means within a same row along same test with different letter differ statistically ($p < 0.05$); Control represents the MRS agar without any potential probiotic

In the case of growth over time, the treatments, except for C. mushroom in hours 0 and 4, showed no significant differences, which demonstrates an inhibitory effect produced by the induced biliary stress in similarity to L. acidophilus. The lethal and sublethal doses are not specified for this microorganism, but it is reported by Wu et al. (2009) that L. casei has the capacity to grow at concentrations of 1.5%, therefore the 0.3% concentration of bile salts used in the experiment, even

though being lower than the concentration in which the probiotic strain is capable to grow, could have affected its ability to grow and multiply, due to its sublethal level as explained previously.

In the case of the effect of the treatments on biliary stress, with the exception of C. mushroom, there were no significant differences in regard to the control, this shows that the potential prebiotics had no effect on the stress generated by the bile on *L. casei*, this can be associated to the composition of the potential prebiotics used, where the complex molecules of high molecular weight prevail as opposed to those of low molecular weight described as characteristics of the prebiotics (Xiao Wang et al., 2015). The results obtained in C. mushroom could be related with and error in sampling at hour 0 and 4.

Conclusions

The biliary stress generated into *L. casei* and *L. acidophilus* in the presence of all the substrates, shows a behavior of sublethal doses in which the bacteria did not die, despite that, the population of bacteria did not report significant growth over time; the control had the same behavior as the treatments, therefore, the study indicates that the potential prebiotics had no effect on the biliary stress resistance.

The literature review included 64 documents of which 11 did not meet the selection criteria. The topic of probiotics and prebiotics was structured in relation to human microbiomes, in addition to the commonly used in vitro evaluation methodologies.

Recommendations

The probiotic potential of C. fungus should be evaluated in periods of time greater than eight hours to demonstrate its possible prebiotic effect, while increasing the number of repetitions to reduce experimental errors.

To fully understand the potential prebiotic effect of Carao, Teosinte, Ceasar´s mushroom, and South American palm weevil, it is necessary to assess their effects on a wider variety of probiotic strains to fully understand the potential benefits of prebiotics.

For future iterations of this study, it is imperative to conduct replicate measurements for each experimental condition.

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