CHAPTER

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Flavonoids effects on the proliferation and protection of Lactobacillus rhamnosus

https://doi.org/10.56238/alookdevelopv1-052

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ABSTRACT

Intestinal microbiota plays an important role in the human organism homeostasis and it is subject to several perturbations caused by diet and environmental factors, inducing dysbiosis. The search for compounds can help rebuild the intestinal microbiota because sometimes this is the only treatment option for intestinal dysbiosis. Phenolic compounds are neutralizing free radicals, showing a mutual relationship with foods rich in flavonoids, so they might be helpful during the restoration of the intestinal microbiota. This study analyzed the direct effect of these compounds on Lactobacillus rhamnosus, cultivated with and without flavonoids in MRS broth, in different growth phases and concentrations. After that, were exposed to enzymes that are involved in the human digestive process to analyze any alteration. Flavonoids quercetin, rutin, hesperetin, and hesperidin were individually tested. The results showed that the presence of flavonoids increased the number of colony-forming units (CFU) compared to the control group (without flavonoids), especially when added before the lag phase. In the digestibility test, quercetin showed a minimal effect for the acidic stomach stage, but for the other steps mimicking the further digestive system, there was no difference compared to the control, all being killed after bile salts. Therefore, we concluded flavonoids are helpful in the proliferation of the tested stain; however, it is necessary to develop more efficient methods so that the flavonoids can overcome the digestive system and reach the intestine with total efficiency.

Keywords: Flavonoids, probiotics, prebiotics, dysbiosis, microbiology, molecular biology.

1 INTRODUCTION

The human body is colonized by several microorganisms during life, including bacteria, archaea, viruses, and eukaryotes, in a complex ecosystem that coexists with its host in a mutualistic way. These microorganisms settle on body surfaces, such as skin and urogenital, respiratory, and gastrointestinal tracts [1,2]. The gastrointestinal tract, especially the small intestine, stands out with conditions for the proliferation of these organisms, called probiotics, constituting intestinal microbiota [3]. Probiotics are defined as live microorganisms that when administered properly and consumed safely, confer benefits to the health of their host and studies show the species to use for treatments [4,5]. Azad and collaborators revealed that the *Bifidobacterium* and *Lactobacillus* genera have the largest number of species, being the most used in research [6]. The small intestine provides nutrients and favorable conditions for probiotics proliferation, such as temperature and anaerobiosis. In turn, intestinal microbiota metabolism assists the fermentation of carbohydrates, vitamin synthesis, increasing intestinal permeability, and epithelial defense mechanism to form themucosal barrier [7,8].

Intestinal microbiota health is intrinsically related to the host health, as shown in germ-free animal studies, that the absence of these microorganisms made them more susceptible to the development of diseases, such as obesity, type II diabetes, hypertension, and inflammatory bowel diseases [9-13]. Scheperjans and coauthors observed that the abundance of the *Prevotellaceae* family has a protective effect on human Parkinson's disease, while its decrease may be a biomarker for this disease [14]. The maintenance of microbiota is essential for host homeostasis, which can contribute during the treatment of several diseases. Compounds that help probiotics proliferate in a safe and lasting way may be the key to restoring the altered microbiota, in addition to being able to optimize foods that supplement the probiotics [15,16].

Conjugation between probiotics and flavonoids was described as mutualistic in the review by Cardona and coauthors, who reported that some flavonoids are absorbed in the small intestine after being metabolized by specific bacteria, and on the other hand, the intake of foods rich in flavonoids modulates probiotic populations, contributing to their maintenance, stimulating the growth of beneficial bacteria, inhibiting the proliferation of pathogenic bacteria [17]. Flavonoids are secondary metabolites of plants, found in leaves, fruits, and seeds, present in the human diet. Its structure has two benzene rings linked to a pyran ring, rich in hydroxyls and electron donor, which confers antioxidant activities, widely studied, by reacting and inactivating superoxide anions, singlet oxygen, lipid peroxide radicals and/or stabilizing free radicals involved in the oxidative process through hydrogenation or complexation with oxidizing species [18,19]. Biologically, the diversity of known molecules plays an important hepatoprotective, antibacterial, anti-inflammatory, anticancer, and antiviral role [20].

Several studies investigated the relationship between foods rich in polyphenols and probiotics, such as cocoa derivatives, grape derivatives, red pitaya pulp, and berries, which have shown significant

promise in optimizing probiotics, functioning as prebiotics [21-24]. Given the evidence presented, the combination of flavonoids and probiotics shows promise and can expand knowledge of how this complex ecosystem works and interacts with the host. In addition, these studies show evidence of how the body can reestablish homeostasis when subjected to changes. This study tested the prebiotic effect of these molecules on the isolated condition of theprobiotic present in the human intestine microbiota and its effect on simulated gastrointestinal digestion.

2 MATERIALS AND METHODS

2.1 STAIN AND INOCULUM PREPARATION

The stain was cultivated in MRS broth (Man, Rogosa & Sharpe, from Kasvi, São José dos Pinhais, PR, Brazil) at 37 °C overnight (14-16 hours) until reach stationary phase, with DO 1.8-2.0, previously determinate in this study and compared with the literature [25]. All tests and controls were serially diluted in saline solution 0,9% (0,9 g/mL NaCl), plated on MRS Agar (from Kasvi, São José dos Pinhais, PR, Brazil), and grew anaerobically for 24 h at 37 °C. After incubation time, bacterial colonies were counted and compared with the control, expressed in percentage. Error percentage was calculated through the ratio between the means and standard deviation of the control and tests.

2.2 MOLECULE CHARACTERISTICS AND EFFECTS ON PROBIOTIC GROWTH

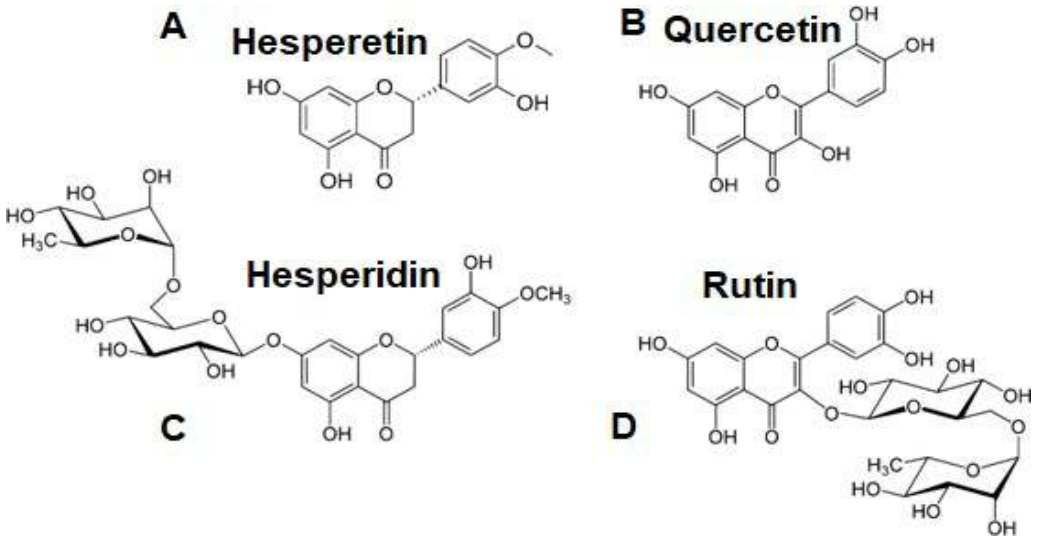
Flavonoids used in this study were Quercetin (QUE), Rutin (RUT), Hesperidin (HET), and Hesperidin (HED), all purchased from Sigma-Aldrich, St. Louis, MO, United States, with ≥ 95% purity. RUT is derived from QUE, as well as HED derived from HET, with both molecules increased with carbohydrates rhamnose and glucose (Figure 1). The stock solutions of 5 mM were prepared in 99,9% DMSO (Dimethyl sulfoxide, from Sigma-Aldrich, St. Louis, MO, United States) using the molar absorption coefficients of 21.880 M-1 cm-1 (375 nm), 18.800 M-1 cm-1 (358 nm), 33.232 M-1 cm-1 (290 nm) and 18.170 M-1 cm-1 (295 nm), respectively, and determinate by UV–Vis Spectrophotometer (BioMate™ 3S, ThermoFisher, USA), equipped with 1.0 cm quard cells of path length, on the range of 250-500 nm absorption spectra.

DMSO was tested separately as solvent control at the range of 0.1% and 1.0% on absolute concentration (99,9 %), and flavonoids were tested on low (10 µM) and medium (50 µM) concentrations. Solvent and phenolic compounds were added in MRS broth for 2 hours on the stationary phase, after growth for 16 hours anaerobically, for analyzing the effect between the concentrations. The medium concentration was added before the lag phase and allowed growth for 16 hours for analyzing the influence during proliferation.

2.3 PRELIMINARY SIMULATION OF GASTROINTESTINAL DIGESTION

The gastrointestinal simulation process was carried out in stages, using digestive enzymes (Sigma, St. Louis MO, USA), under constant agitation. The pH of each solution stage was adjusted using 1M HCl or 1M NaHCO3. To simulate mastication, 1.2 mL/min of salivary solution (0.33 mg of α-amylase/mL of 1 mM CaCl2) was added at neutral pH of 6.9, under intense agitation (200 rpm), for 2 minutes. For the esophagus-stomach step, stirring at 130 rpm and pepsin solution (25 mg/mL of 0.1 M HCl) was added at a rate of 0.05 mL/mL of inoculum and pH 2.0 for 90 min. The intestinal solution was prepared with 2 g/L of pancreatin and 12 g/L of bile salts, diluted in a 0.1 M NaHCO3 solution, stirred at 45 rpm, with pH 5.0 for 30 min. Finally, the pH was adjusted to 6.5 with NaHCO3 to simulate the ileal portion for 60 min [26,27]. After each step, a 100 µL-aliquot was serially diluted in saline solution and plated on MRS agar for 24 hours.

Figure 1. Chemical structure of (A) hesperetin, (B) quercetin, (C) hesperidin, and (D) rutin.



3 RESULTS

The first test was with DMSO, added at a rate of 0.1% (1 µL/mL of MRS broth) and 1.0% (10 µL/mL of MRS broth) for 2 hours on the stationary phase, after growth overnight. The percentage of growth increased to 32% and 69%, respectively. When 1.0% was added before the lag phase and growth anaerobically overnight, the CFU was increased to 74%.

Phenolic compounds used in this study also increased the CFU, overcoming the DMSO effect. The difference between low and medium concentration was not so significant when added in the stationary phase after growing overnight, resulting, respectively, in an increase of 60% and 78% for quercetin, 98%, and 106% for rutin, 57% and 78% for hesperetin, and 126% and 134% for hesperidin.

However, when medium concentrations were added before the lag phase and growth overnight, large increases occurred compared to the control, with hesperidin having the greatest increase (318%), followed by

rutin (237%), hesperetin (131%), and quercetin (101%). All percentages are expressed and compared in figure 2.

The preliminary simulation of gastrointestinal digestion was done with quercetin. The results showed that the molecule increased the formation of colonies in the stages of the mouth and stomach, and in the most acidic step, the stomach, its percentage increased by 426% (Figure 3).

4 DISCUSSIONS

DMSO is a widely used solvent due to its efficient physicochemical properties in solubilizing water-insoluble compounds, such as flavonoids. However, a high concentration of this solvent is toxic and may kill cells [28]. Then the solvent was tested as a control, and the results show that DMSO increased CFU in three treatments. The increase in CFUs may be a result of the strain's ability to metabolize the solvent and use it as a carbon source, as explained by Vdihya & Thatheyus in their study with DMSO and Bacillus subtilis [29]. Hesperidin and rutin had the highest increase, which might be related to the carbohydrates in the molecules, also being metabolized by bacteria as a source of carbon.

Figure 2. Effect of the presence of flavonoids on colony-forming units. Relation between the low and medium concentration on the percentual when added during the stationary phase. When the flavonoids were added before the lag phase, at medium concentration the percentual was intensely increased.

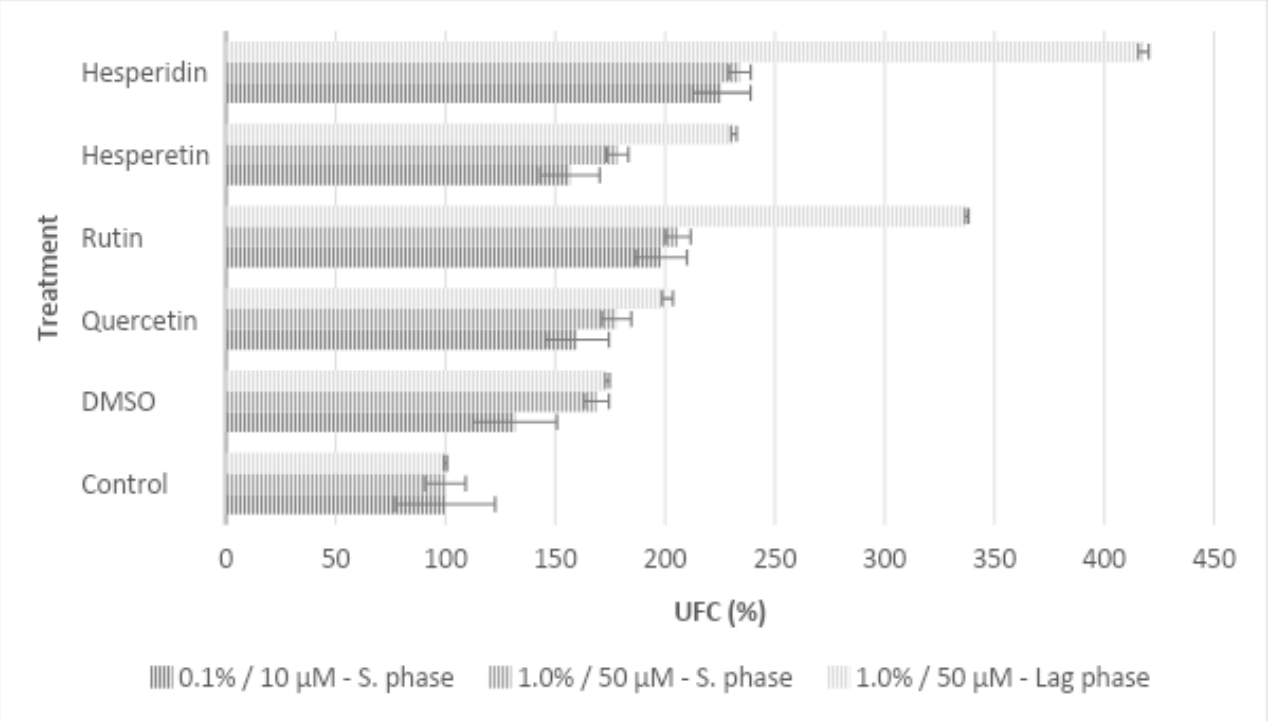
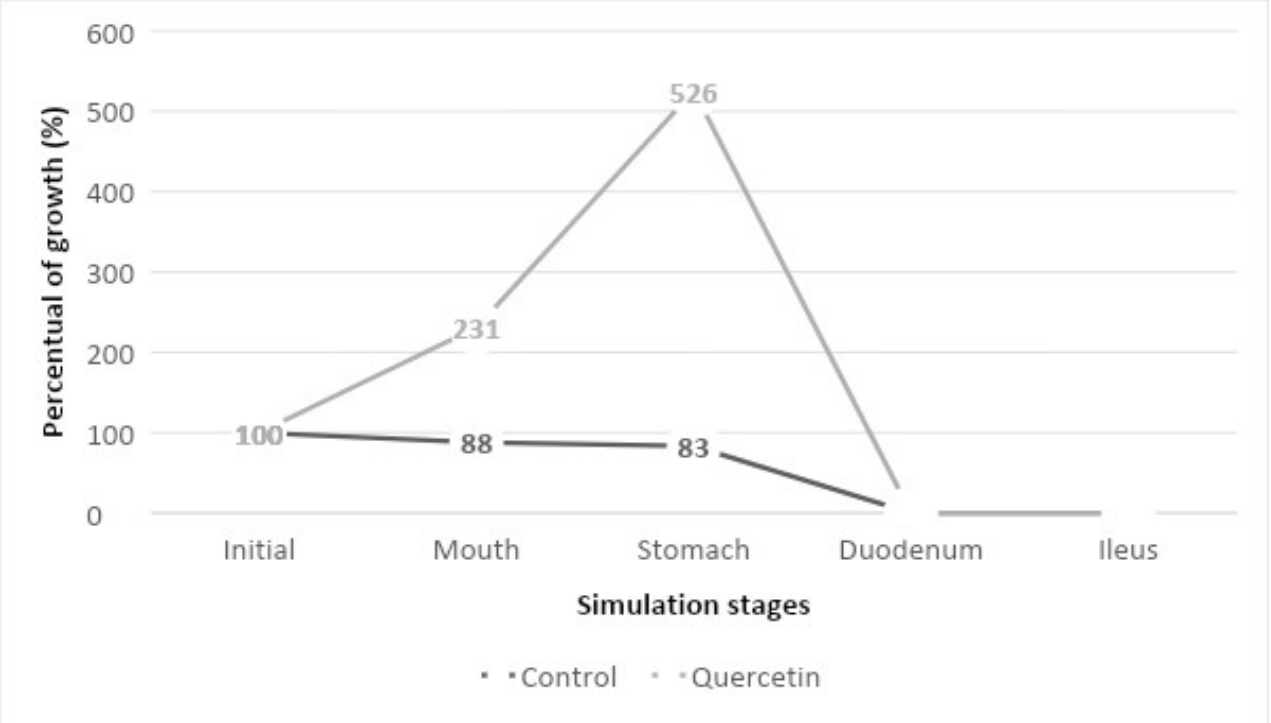


Figure 3. Simulation of gastrointestinal digestion. Comparison strain treated with quercetin and a significant increase in colony-formed units, during the control remained constant until the addition of bile salts in the duodenal stage.



Studies have shown how foods rich with flavonoids, this study has notable effects on the intestinal microbiota, such as quercetin, which stands out with its protective action against intestinal dysregulation during the use of antibiotics, increased intestinal permeability associated with the production of acids short-chain fatty acids, in addition to acting against pathogens, promoting balance in the microbiome [30-32]. Hesperidin proved to be effective in increasing populations of *Bifidobacterium* by influencing the action of IgA in mice [33]. Rutin action depends on how it's metabolized, producing different types of phenolic acids that can help in the bioavailability of other compounds [34], consolidating its prebiotic potential for these microorganisms and other benefits to the host.

The increase during the stomach stage might be related to the molecule propriety of the electron donor, which confers antioxidant activities [35]. This stage at control confirmed the tolerance of *L. rhamnosus* to acid pH [36,37], keeping colony-forming units close to 100%.

However, *L. rhamnosus* is also described as bile tolerant, and bile salts killed all cells in the duodenum stage. This result might be from its biological function in the digestion of lipids, damaging cell membranes and restricting the growth of some species of bacteria [38].

5 CONCLUSIONS

In conclusion, flavonoids presented an effect on the proliferation of the L. rhamnosus strain. This effect is even more potentiated when added medium before the proliferative phase, reaffirming the prebiotic effect of these compounds. The combination of probiotics and flavonoids is promising and can expand knowledge about how the microbiota works and your applications in several diseases

of the intestinal tract. Preliminary tests showed that these molecules might amplify proliferation in acid pH, but would not protect from all digestion stages.

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