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Evaluation of the antimicrobial potential of digested and undigested carob phenolic extracts: Impact on selected gut microbiota

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ABSTRACT

Carob pulp is a natural source of polyphenols, which have been shown to possess health benefits. These compounds play a crucial role in initiating, shaping, and modulating the gut microbiota. The objective of this study was to evaluate the impact of carob pulp phenolic extracts on nine specific groups of human gut microbiota before and after *in vitro* gastrointestinal digestion. The effects of pure gallic and coumaric acids were also tested. The results showed that the treated phenolic compounds exhibited inhibitory effects on

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the growth of most pathogenic bacteria. Gallic acid, in particular, demonstrated the most potent antimicrobial effect on *Listeria monocytogenes*, reducing its growth to below 5%. *Staphylococcus aureus* and *Escherichia coli* showed a growth reduction of up to 10%. Furthermore, both phenolic acids, before and after digestion, led to a slight reduction in *E. coli* O157:H7 numbers. Probiotic bacteria experienced minimal decrease following exposure to phenolic extracts. However, the growth of *Lactobacillus casei* ssp. *rhamnosus* was significantly inhibited by almost 50%. Interestingly, the *in vitro* digestion process exhibited a stronger antibacterial effect against pathogenic bacteria compared to probiotic bacteria. These results highlight the potential of carob phenolic extracts in modulating the intestinal microbiota, thereby offering interesting prospects for the development of diet-based health strategies.

1. INTRODUCTION

Polyphenols have been recognised for their potential beneficial effects on human health. Several studies have shown that the diets rich in fruit and vegetables are associated with a reduced risk of chronic diseases such as cardiovascular diseases, specific cancers, and neurodegenerative diseases (Etxeberria et al., 2013; Chaalal et al., 2016).

Carob is an essential source of phenolic compounds, which include phenolic acids, flavonoids, condensed tannins, and hydrolysable tannins. During digestion, carob phenolic extract undergoes interactions with the gut microbiota and digestive enzymes, which can lead to the transformation of some phenolics into smaller metabolites, affecting their bioavailability and potential health benefits (Ydjedd et al., 2017).

Polyphenols and their metabolites have been found to modulate the intestinal ecology by affecting the gut microbiota (Selma et al., 2020). In fact, several phenolics have been identified as potential antibacterial compounds suppressing pathogenic bacteria in the human gut (Lee et al., 2006).

The microbiome is increasingly recognised as playing a significant role in health and disease, with implications for clinical problems such as frailty in the elderly, inflammatory bowel disease, irritable bowel syndrome, colorectal cancer, and gut-derived infections (Flint et al., 2012).

In vitro digestion models used to study the modulation of the gut microbiota by polyphenol consumption are simplified systems with shorter digestion durations compared to real transit times. Additionally, these models may not fully account for the influence of complex food matrices on polyphenol metabolism and biological activity. However, the investigation of the effects of various molecules and extracts, including phenolic compounds, *in vitro* on gut microbiota is of great interest (Lee et al., 2006; Sousa et al., 2006; Bosscher et al., 2009; López-Nicolás et al., 2014). Building upon our previous study focused on the effects of *in vitro* gastrointestinal digestion on phenolic compounds of carob (*Ceratonia siliqua* L.) pulp extracts and their antioxidant capacity (Ydjedd et al., 2017), current research explores the interactions between phenolic compounds and intestinal microbiota in a bidirectional manner (Dias et al., 2021), as well as studies the development of the microbial world after *in vitro* digestion and fermentation, which contributes to the bioactivity of polyphenols (Plamada and Vodnar, 2021).

Therefore, the aim of this study is to investigate the effects of digested and undigested carob phenolic extracts on selected groups of human intestinal microbiota, including probiotics, commensals, and pathogens.

2. MATERIALS AND METHODS

2.1. Chemicals

The origin and brand of different enzymes and reagents used in this study were reported in the study of López-Nicolás et al. (2014).

2.2. Sample preparation

A quantity of 10 g of fresh carob (*C. siliqua* L.) pulp of the *Lahlou* variety (ripe stage), typically cultivated in the Bejaia area of Algeria, was added to 100 mL of acetone/water (70:30, v/v). After homogenisation, centrifugation, filtration, and lyophilisation, the dry extract was stored at 4 °C until use (Ydjedd et al., 2017).

2.3. *In vitro* gastrointestinal digestion

The *in vitro* gastrointestinal digestion method was carried out following the method described in our previous study Ydjedd et al. (2017).

2.4. Bacterial strain and culture conditions

The sources and characterisation of bacterium strains used in this study were described by López-Nicolás et al. (2014).

2.5. Effect of extracts on intestinal bacteria growth

Digested and undigested phenolic carob pulp extracts (ripe stage) were tested at concentrations of 0.6, 1.25, 2.5, 5, and 10 mg mL^{-1}. Gallic and coumaric acids, dissolved in DMSO, were used as controls. The antimicrobial activity of all samples against selected bacterial strains was assessed following the protocol described by López-Nicolás et al. (2014).

2.6. Statistical analysis

The results were presented as mean ± standard deviation. Statistical analysis was conducted using Statistica 5.5 software (StatSoft Inc., USA). Likewise, the Principal Components Analysis (PCA) was performed using the XLSTAT software (Version 2009.1.01, Addinsoft®).

3. RESULTS AND DISCUSSIONS

3.1. Effect of phenolic extracts and the control on pathogenic bacteria growth

Figure 1 illustrates the effect of different phenolic carob concentrations and controls (gallic and coumaric acids) on various pathogenic bacteria growth. In general, the findings indicate that all concentrations of the digested and undigested extracts, as well as the controls, had a significant
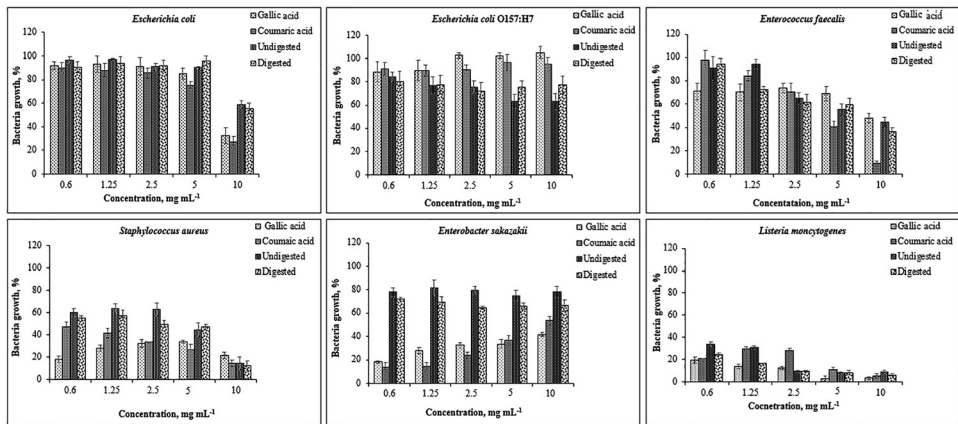
Fig. 1. Effect of digested and undigested phenolic extracts from carob pulp and control (gallic and coumaric acids) at different concentrations on the growth of various pathogenic bacteria

effect on bacterial growth, especially at the concentration of 10 mg mL^{-1}. Except for *Escherichia coli* (EC), concentrations of 0.6, 1.25, 2.5, and 5 mg mL^{-1} of extracts and controls revealed no significant effect on the growth of this bacterium. From the concentration of 0.6–5 mg mL^{-1}, the values ranged between 74.73 and 91.99% for controls and between 90.33% and 97.22% for undigested and digested extracts. At the concentration of 10 mg mL^{-1}, the results revealed a significant inhibitory effect on EC growth, with values of 32.47, 27.32, 55.27, and 58.93% for the effect of gallic acid, coumaric acid, digested, and undigested extracts, respectively.

For *E. coli* O157:H7, the results revealed a minimal impact of the different concentrations of extracts and controls on bacterial growth. Indeed, *E. coli* O157:H7 growth ranged between 88.63 and 105.18% under the effect of the controls, between 72.02 and 80.43% under the effect of undigested extracts, and between 62.99 and 84.08% for digested extract.

Concerning *Enterococcus faecalis*, *Staphylococcus aureus*, and *Listeria monocytogenes* the results showed that the growth of these bacteria significantly decreased with the increase of digested, undigested, and the control concentrations. Indeed, *L. monocytogenes* decreased from 19.44 to 3.51%, from 20.97 to 5.43%, from 33.55 to 9.06%, and from 24.78 to 6.04% under the effect of gallic acid, coumaric acid, undigested extract, and digested extract, respectively, by the increasing the concentration of extracts (digested and undigested) and the control from 0.6 to 10 mg mL^{-1}.

Furthermore, the results revealed that increasing the control concentrations (gallic and coumaric acids) from 0.6 to 10 mg mL^{-1}, the growth of *Enterobacter sakazakii* increased from 18.17 to 41.72% under the effect of gallic acid and from 13.73 to 53.65% under the effect of coumaric acid. Likewise, no significant difference was observed in *E. sakazakii* growth with increasing extract concentrations, with values ranging from 75.18 to 81.45% and from 64.92 to 71.98% for the effect of undigested and digested extracts, respectively.

3.2. Effect of phenolic extracts and controls on the growth of probiotic bacteria

The effects of different phenolic extracts and controls on probiotic bacteria are presented in Fig. 2. The results show a significant decrease of the growth of *Lactobacillus casei*
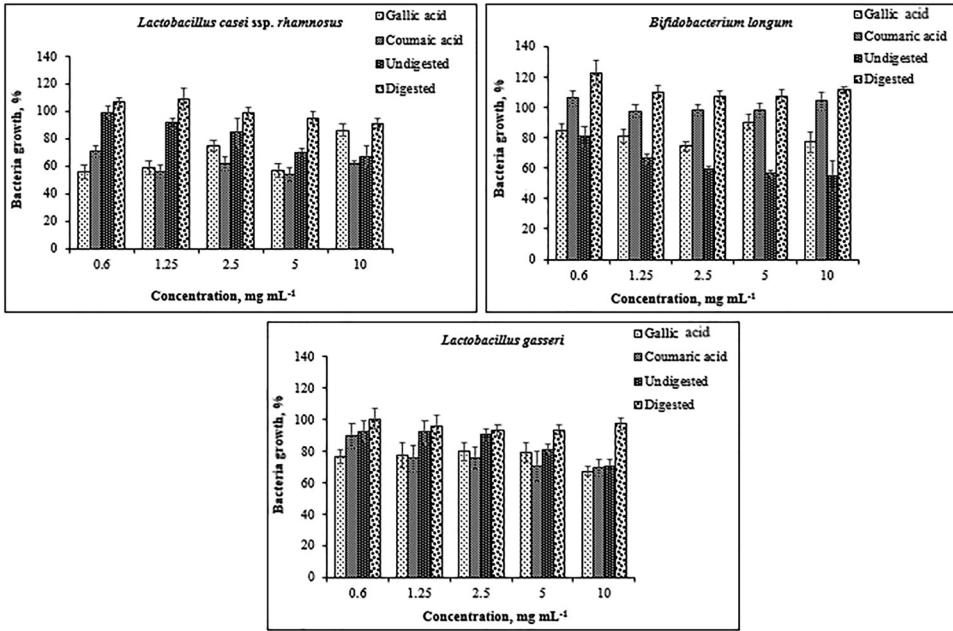
Fig. 2. Effect of digested and undigested phenolic extracts from carob pulp and controls (gallic and cou-
maric acids) at different concentrations on the growth of various probiotic bacteria

ssp. *rhamnosus*. As the extracts' concentrations increased from 0.6 to 10 mg mL^{-1}, the growth of
bacteria decreased from 99.29 to 67.02% and from 106.51 to 91.33% for the effect of undigested
and digested extracts, respectively. An increase in the growth of *L. casei* ssp. *rhamnosus*
from 55.51 to 86.28% was observed with the increase in gallic acid concentration from 0.6 to
10 mg mL^{-1}. In addition, no significant effect was observed for coumaric acid with values
ranging from 71.01 to 62.10%.

Regarding *Bifidobacterium longum*, its growth decreased significantly from 84.66 to 77.01%
for gallic acid and from 81.24 to 55.23% for undigested extracts when their concentrations
increased from 0.6 to 10 mg mL^{-1}. Furthermore, a slight decrease from 122.97 to 111.47%
was observed for digested extract. Likewise, no significant difference was observed for coumaric
acid with values ranging from 97.47 to 106.09%.

The results indicated also a significant decrease in *Lactobacillus gasseri* growth from 76.38 to
66.99%, from 89.46 to 69.58%, and from 92.27 to 70.62% when increasing the concentrations
from 0.6 to 10 mg mL^{-1} of gallic acid, coumaric acid, and undigested extracts, respectively.
A slight decrease of *L. gasseri* growth with values ranging from 100.27 to 96.98% was observed
for the digested extract.

3.3. Correlation between the different concentration of extracts and bacteria growth

The correlation between the concentrations of the controls and extracts (digested and undi-
gested) and the growth of selected bacteria is presented in Table 1. For the pathogenic bacteria,

Table 1. Correlation between extracts concentrations, mg mL^{-1} and bacteria growth, %

	Pathogenic bacteria					
	Escherichia coli		*Escherichia coli* O157:H7		*Enterococcus faecalis*	
Substances	Correlation coefficient (r)	Equation	Correlation coefficient (r)	Equation	Correlation coefficient (r)	Equation
Gallic acid	$r = -0.941$	y = 6.4389x + 103.76	$r = 0.921$	y = 4,5691x + 84,029	$r = -0.713$	y = 4.7217x + 80.648
Coumaric acid	$r = -0.973$	y = 6.7118x + 99.188	$r = 0.784$	y = 1.5818x + 88.023	$r = -0.980$	y = 22.161x + 127.01
Undigested	$r = -0.957$	y = 3.9996x + 102.39	$r = -0.955$	y = 5.6248x + 89.44	$r = -0.952$	y = 13.199x + 109.56
Digested	$r = -0.847$	y = 3.7802x + 100.17	$r = -0.435$	y = 0.84x + 79.193	$r = -0.966$	y = 12.883x + 103.64
	Staphylococcus aureus		*Enterobacter sakazakii*		*Listeria monocytogenes*	
Gallic acid	$r = 0.304$	y = 1.2822x + 22.889	$r = 0.968$	y = 5.2822x + 14.889	$r = -0.958$	y = 4.2272x + 23.1
Coumaric acid	$r = -0.991$	y = 8.1244x + 57.25	$r = 0.957$	y = 10.289x – 2.4076	$r = -0.747$	y = 4.9599x + 33.864
Undigested	$r = -0.832$	y = 10.998x + 82.201	$r = -0.349$	y = 0.5774x + 80.33	$r = -0.881$	y = 7.1547x + 39.591
Digested	$r = -0.826$	y = 9.5648x + 73.051	$r = -0.771$	y = 1.3867x + 71.956	$r = 0.707$	y = 2x – 4
	Probiotic bacteria					
	Lactobacillus casei ssp. rhamnosus		*Bifidobacterium longum*		*Lactobacillus gasseri*	
Gallic acid	$r = 0.699$	y = 5.9354x + 48.698	$r = -0.141$	y = 0.5665x + 83.16	$r = -0.532$	y = 1.7142x + 80.959
Coumaric acid	$r = -0.482$	y = 2.0381x + 67.085	$r = -0.112$	y = 0.2848x + 101.67	$r = -0.884$	y = 4.4618x + 89.39
Undigested	$r = -0.986$	y = 8.5945x + 108.3	$r = -0.915$	y = 6.2059x + 82.307	$r = -0.915$	y = 5.4651x + 101.72
Digested	$r = -0.937$	y = 4.3785x + 113.21	$r = -0.636$	y = 2.5699x + 119.69	$r = -0.457$	y = 0.8688x + 98.411

the results indicated a strong positive correlation between the gallic and coumaric acids concentrations and EC. O157:H7 growth with correlation values of $r = 0.921$ and $r = 0.784$, respectively. Similarly, a positive correlation was observed between the gallic and coumaric acids and *E. sakazakii* growth with values of $r = 0.968$ and $r = 0.957$, respectively, as well as between digested extracts and *L. monocytogenes* growth with a correlation value of $r = 0.707$. Likewise, a weak correlation was observed between gallic acid and *S. aureus* growth with a correlation value of $r = 0.304$. On the other hand, negative correlations were found between the other extracts and the tested bacteria, with correlation values ranging from $r = -0.991$ (between coumaric acid and *S. aureus*) to $r = -0.349$ (between undigested extract and *E. sakazakii*).

For the probiotic bacteria, a positive correlation was observed between gallic acid and *L. casei* ssp. *rhamnosus*, with a correlation value of $r = 0.699$. Negative correlations were observed between the digested and undigested extracts as well as the control and the three tested bacteria, with correlation values ranging from $r = -0.986$ (between undigested extract concentrations and *L. casei* ssp. *rhamnosus*) to $r = -0.112$ (between coumaric acid and *B. longum*).

3.4. Principal components analysis (PCA)

Figure 3 presents the biplots of the PCA of carob phenolic extracts and controls at different concentrations (0.6, 1.25, 2.5, 5, and 10 mg mL^{-1}). The PCA plots accounted for 89.41%, 86.24%, 89.01%, 83.90%, and 76.76% of the variability in the dataset, respectively.

The results indicated that a concentration of 0.6 mg mL^{-1} of gallic and coumaric acids resulted in very low growth rates for all tested bacteria except for E.C. and E.C. O157:H7, which exhibited a high growth rate. After digestion, it became evident that the growth rate of beneficial bacteria (*L. casei* ssp. *rhamnosus*, *L. gasseri*, and *B. longum*) increased significantly. However, the growth of pathogenic bacteria such as *S. aureus* and *E. faecalis*, was slightly inhibited resulting in moderate growth rates. When the concentration of these compounds increased to 1.25 mg mL^{-1} and after digestion, it was observed that while the E.C. O157:H7 growth slowed down, the growth of the beneficial bacteria (*L. casei* ssp. *rhamnosus* and *L. gasseri*) accelerated exhibiting very high growth rates.

At higher concentrations (>2.5 mg mL^{-1}), the results clearly demonstrated that after digestion, the three beneficial bacteria exhibited remarkably high growth rates, while the pathogenic bacteria a decline most notably for *Listeria moncytogenes*. These findings are very promising and suggest potential positive effects of the digested extract.

3.5. Discussions

The effects of phenolic compounds on the intestinal microbiota are attributed to their influence on the growth, metabolism, and membrane functioning of bacterial cells (Plamada and Vodnar, 2021). In this study, the carob pulp phenolic extract exhibited inhibitory effects on most pathogenic bacteria compared to probiotic bacteria. Indeed, gallic acid demonstrated the strongest antimicrobial effect on *L. monocytogenes*, and both phenolic compounds reduced *S. aureus* and *E. faecalis* growth. Previous studies have also highlighted the inhibitory effects of gallic acid on Gram-positive bacteria (Rua et al., 2011; López-Nicolás et al., 2014), while cinnamon, known for its richness in coumaric acid, has shown significant effects against Gram-positive bacteria such as *S. aureus* (Nazhand et al., 2020; Krautkramer et al., 2021). Studies on red wine polyphenols have shown changes in the *E. faecalis* growth (Cueva et al., 2017; Gil-Sánchez et al., 2018). Phenolic compounds have also exhibited inhibitory activity against *E. sakazakii* and *E. coli*, as reported by Requena et al. (2010). The observed differences in the growth of pathogenic bacteria, especially *L. monocytogenes*, could be attributed to several factors, including variations in culture preparation, differences in strain response, and environmental factors such as temperature and pH, nutrient availability, and oxygen levels. Small differences in these conditions between experiments could impact the observed growth inhibition.

Regarding probiotic bacteria, the inhibitory effects of phenolic compounds were not significant against tested lactic acid bacteria. *B. longum* and *L. gasseri* showed minimal reductions, while a limited antimicrobial effect of phenolic compounds, not exceeding 50%, was
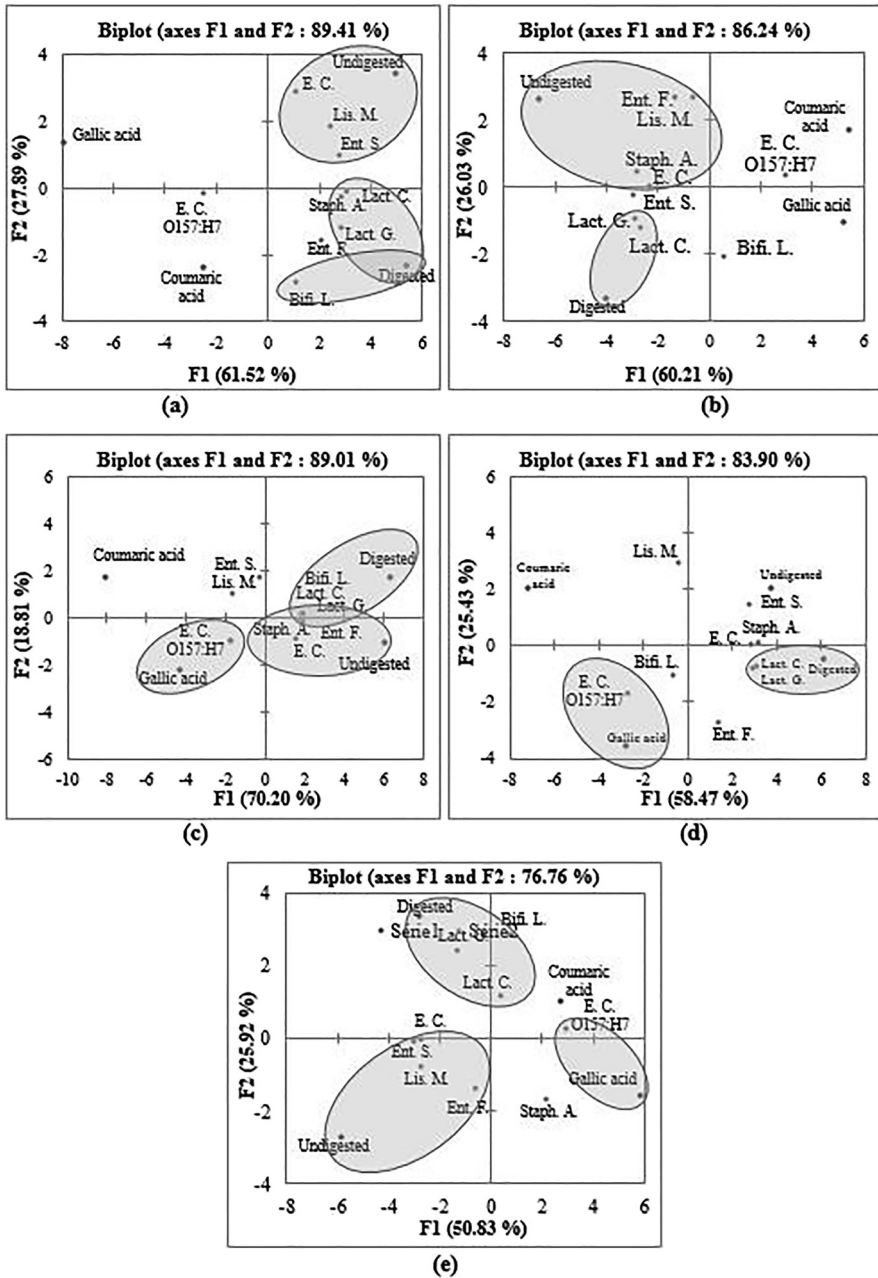
Fig. 3. Principal component analysis of the effect of carob phenolic extract (digested and undigested) and controls (gallic and coumaric acids) at different concentrations on the growth of pathogenic and probiotic bacteria. (**a**): 0.6 mg mL^{-1}, (**b**): 1.25 mg mL^{-1}, (**c**): 2.5 mg mL^{-1}, (**d**): 5 mg mL^{-1}, (**e**): 10 mg mL^{-1}

observed for *L. casei* ssp. *rhamnosus*, which is particularly sensitive to phenolic compounds (Puupponen-Pimiä et al., 2001). Generally, probiotic bacteria exhibit higher resistance to polyphenols compared to pathogenic bacteria (Parkar et al., 2008). Coumaric and gallic acid have been shown to promote the growth of bifidobacteria and lactobacilli, while phenolic acids have properties that improve the growth of probiotic bacteria and gut microbiota (Gowd et al., 2019; Liu et al., 2019; Dias et al., 2021). These findings are consistent with the observations of Pozuelo et al. (2012), who reported that a phenolic extract from red grapes or grape seeds had several advantages on *L. gasseri* growth.

Furthermore, it was observed that most pathogenic bacteria had increased growth rates, except for *E. coli* O157:H7, which experienced a slowdown in its development. On the other hand, probiotic bacteria exhibited higher growth rates. These findings align with the research conducted by Sáyago-Ayerdi et al. (2021). Moreover, another *in vitro* study demonstrated that the addition of red wine polyphenols exerted an antimicrobial effect on the intestinal microbiota (Barroso et al., 2014).

The *in vitro* digestion leads to bioconversion of phenolic compounds into smaller metabolites, such as phenolic acids. In fact, the increase in naringenin concentration after digestion of carob pulp extracts can be attributed to the transformation of apigenin into naringenin, as reported by Hanske (2009). To better understand this bioconversion, *in vivo* studies using animal models or human trials are necessary. These studies can provide insights into the complex interactions between phenolic compounds, gut microbiota, and host factors, which play a critical role in determining the ultimate bioactivity and health effects of these compounds.

4. CONCLUSIONS

The phenolic compounds present in carob pulp (before and after digestion) have demonstrated beneficial effects on the intestinal microbiota, specifically on both pathogenic and probiotic bacteria. In the case of pathogenic bacteria, gallic acid has shown the most effective inhibitory effect, reducing the growth of *L. monocytogenes* to below 5%, while *S. aureus* and *E. coli* experienced a growth reduction of up to 10% at known concentrations. Likewise, *E. faecalis* growth was reduced up to 30%. furthermore, *E. coli* O157:H7 growth was only slightly inhibited by digested and undigested extracts. On the other hand, lactic acid bacteria, which include probiotic bacteria, were generally unaffected by the phenolic compounds. As a result, the growth of probiotic bacteria was only slightly reduced, and the digestion process exhibited a more pronounced antimicrobial effect on pathogenic bacteria compared to probiotic bacteria.

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