

Article

Biochar's Adsorption of *Escherichia coli* and Probiotics *Lactiplantibacillus plantarum* and *Limosilactobacillus reuteri* and Its Impact on Bacterial Growth Post In Vitro Digestion

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Featured Application: The present study highlights the potential of biochar, obtained from vine pruning biomass, as a functional ingredient in animal feed. Biochar has been shown to exhibit inhibitory activity against pathogenic strains of *E. coli* and to promote the growth of probiotic bacteria such as *Lactobacillus* sp. These biochar properties can be exploited in the agro-livestock field, where they can help improve animal intestinal health, reduce gastrointestinal diseases, and contribute to the development of sustainable agricultural practices. Notably, the functional properties of biochar are maintained following in vitro digestion, indicating the potential efficacy of biochar for in vivo applications.



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Abstract: Background: Biochar has gained increasing attention for its potential benefits in improving animal health. Its physical and chemical properties depend on the starting biomass and production technology. This study investigates its functional properties versus bacteria. Methods: The morphology and physical properties of biochar from vine pruning were evaluated by SEM. The adsorption capacity for pathogenic *E. coli* F4+ and F18+ and probiotic microorganisms such as *Lactobacillus* sp. was assessed by plate count after contact with biochar. The growth activity on pathogenic and probiotic bacteria was tested after in vitro digestion. Results: Biochar from grapevine pruning did not maintain the original structure and showed both smooth and rough surfaces. The binding capacity varied across bacterial species. At concentrations of 20 mg/mL, up to 74% of *E. coli* adhered to the biochar surface, while the maximum adsorption rate of *Lactobacillus* sp. was around 38%. An inhibitory activity against *E. coli* (maximum reduction: 35%) and a growth-promoting effect for *Lactobacillus* sp. were observed (maximum promotion: 6%). Conclusions: These findings highlight the potential of biochar as a functional feed ingredient and that its functional properties are preserved after in vitro digestion.

Keywords: biochar; adsorption capacity; functional ingredient

1. Introduction

Biochar is a carbon-rich material derived from the pyrolysis of organic matter. It has gained significant attention in the last ten years due to its sustainability and versa-

tile applications in various fields, including agriculture, remediation, energy production, purification, and, more recently, farming [1]. Biochar production exploits biomasses that have decomposed through thermo-chemical degradation under controlled and limited oxygen conditions, leading to the formation of a highly porous and recalcitrant material [2]. Typically, pyrolysis enables a biochar yield to decrease to within 30% to 60%, with its byproducts such as bio-oil (high energy density liquid) and syngas (low energy density gas) [3]. The temperature of the production process can differ depending on the type of biochar being produced. Typically, temperatures of between 400 and 500 °C produce more char, while higher temperatures (over 600 °C) favor the production of more liquid and gaseous fuels [4]. From a chemical point of view, pyrolysis leads to a biochar that is rich in carbon, hydrogen, oxygen, and nitrogen [5]. The chemical transformation of biomass into biochar includes subsequent reactions such as thermal decomposition, repolymerization, and aromatization of the organic compounds of the original biomass [6]. The inorganic compounds remain as ash. While the chemical composition is crucial in the evaluation of biochar as an innovative material for different applications, its physicochemical properties are equally important. Characteristics such as the surface functional groups, pore structure, redox properties, and cation exchange capacity are significantly influenced by the production conditions (e.g., temperature, heating rate, residence time), which can affect the efficacy of the biochar in specific applications [7]. The unique structure of biochar in terms of surface area and porosity greatly impacts its adsorption ability. These values are important when biochar is used as a functional product because, as reported by Leng et al. (2021), greater surface areas and more porous structures typically enhance the adsorption capacity [8]. Generally, biochar is characterized by surface areas ranging from 8 to 132 m²/g [9].

Biochar is characterized by pores of different sizes. The most abundant pores are 5–20 µm in diameter and are thus suitable for microbial habitation, as the sizes of bacteria range from 0.3 to 3 µm. Mesopores (2–50 nm) are responsible for the capacity to adsorb organic molecules, and micropores (below 2 nm) are generally inaccessible. The percentage of pores that are located on surface areas typically increases the adsorption of microorganisms [8,10]. However, these are not the only characteristics that play a key role in adsorption mechanisms. In fact, on the one hand, the functional groups on the biochar surface (e.g., hydroxyl -OH, carboxyl -COOH, and aldehyde -CHO) interact with microbial cell walls through various mechanisms, such as hydrogen bonding, electrostatic attraction, and hydrophobic interactions [11]. For instance, biochar with a high degree of aromaticity tends to have stronger hydrophobic properties, which can increase the adsorption of hydrophobic microbial species.

On the other hand, the surface charge of biochar also plays a crucial role in adsorbing charged microbial cells. For example, negatively charged biochar surfaces may effectively adsorb positively charged bacteria. In addition, microorganisms can be physically trapped within the pores of biochar, especially if the pore size matches the size of the microbes [12].

All these properties have recently led the scientific community to focus on the interactions between biochar and microorganisms. Several studies have demonstrated that biochar interacts with soil microbiota, which improves soil quality, enhances the nutrient content, and indirectly makes plants more resistant to pathogens [13–15].

The use of biochar has also been explored as a feed ingredient. The results demonstrated a beneficial impact on animal production, and in particular, an improvement in animal health and a reduction in gas emissions. In terms of animal production, macroscopic effects such as zootechnical performance have been evaluated, which may be attributed to its capacity to modulate gut microbiota and influence metabolic processes in animals. Han et al. (2018) [16] reported that the administration of rice straw biochar to rats improved

gut mucosal structure and modified cecal microbial communities. This was accompanied by an increase in the *Firmicutes* to *Bacteroidetes* ratio and an increase in beneficial bacteria, including *Lachnospiraceae* and *Clostridium*.

Given the morpho-structural characteristics of biochar and its functional properties, this study evaluates, in vitro, the capacity of biochar derived from vine pruning biomass to adsorb microorganisms such as pathogenic *E. coli* and probiotic *Lactobacillus* sp. Simultaneously, the potential bioactive effects of biochar on the same microorganisms were investigated when biochar was used as feed by simulating in vitro digestion. The ultimate aim was to explore and understand the potential beneficial effects of biochar and to provide evidence to support the hypothesis that it can modulate gut microbiota when incorporated into animal diets.

2. Materials and Methods

2.1. Materials and Bacteria

Vine biochar (VBC) is a commercial product, which we obtained from the manufacturer, Romagna Campagna, near Ravenna in Italy. It is characterized by a low surface area of 28 m²/g, only mesopores (40 nm), a porosity value of 58%, and a ZPC value of −39 mV. The product was manufactured by a proprietary procedure involving the controlled pyrolysis of vine pruning waste at temperatures of between 450 °C and 500 °C.

The functional groups on the vine-derived biochar were characterized using Fourier-transform infrared (FTIR) spectroscopy. The spectrum revealed residual carboxylate and carbonate groups. Additionally, aromatic vibrational modes and a C–O–C stretching mode—probably originating from cellulose or lignin residues—were also observed, as described in Dotti et al. (2025) [17].

The biochar was ground with a pestle, and the powder was separated by size (below 500 µm). *Escherichia coli*, characterized by fimbriae F4+ and F18+, was obtained from the private collection of the University of Milan (Lodi, Italy). *E. coli* pathotypes were cultured in Luria-Bertani (LB) broth at 37 °C with shaking for twelve hours. *Lactiplantibacillus plantarum* and *Limosilactobacillus reuteri* were obtained from Biotechnologie BT s.r.l. (Todi, Italy). *L. plantarum* and *L. reuteri* were grown in Man Rogosa Sharpe broth (MRS) at 30 °C without shaking for twenty-four hours.

2.2. Scanning Electron Microscopy (SEM)

The samples were placed directly on the stub, sputtered with carbon, and observed under an FE-SEM Sigma (Zeiss, Oberkochen, Germany) at 8 KV and a working distance of 5 mm. The size of the samples and the area and diameter of the pores were measured using ImageJ v. 1.54K. For ImageJ analysis, we manually delimited the pore areas using “polygon selection” to match the line to the pore edge. Scaling was performed automatically when the image was opened.

2.3. Adsorption Test for *E. coli*

Approximately $8\text{--}10 \times 10^6$ *E. coli* F4+ and F18+ cells were inoculated separately in 10 mL of LB broth and supplemented with various concentrations of VB biochar (0, 5, 10, 20 mg/mL). They were incubated at 37 °C with gentle agitation for two hours. The mixtures were then centrifuged at $600 \times g$ for five minutes to remove the solid component (biochar). An aliquot of the supernatant was then serially diluted and plated on LB agar at 37 °C for 14 h to determine the number of bacteria present (CFU/mL). The analysis was performed in biological duplicates, and the CFU was determined using 3 replicates for each dilution tested ($n = 12$) [18].

2.4. Adsorption Test for *L. plantarum* and *L. reuteri*

Approximately $45\text{--}50 \times 10^6$ *L. plantarum* and *L. reuteri* cells were inoculated in 10 mL of MRS broth and different concentrations of VB biochar (0, 5, 10, 20 mg/mL) and incubated at 30 °C with gentle agitation for two hours. The mixtures were then centrifuged at $600 \times g$ for five minutes to remove the biochar. An aliquot of the supernatant was then serially diluted and plated on MRS agar at 30 °C for 48 h in microaerophilic conditions for the enumeration of bacteria (CFU/mL). The analysis was performed in biological duplicates, and the CFU was determined using 3 replicates for each dilution tested ($n = 12$).

2.5. In Vitro Simulated Digestion

Vine biochar was subjected to in vitro digestion following the static INFOGEST official protocol [19]. All the digestion experiments were conducted with the same batch of enzymes. The digestions were conducted using one gram of sample. The system included three sequential phases (oral, gastric, and intestinal). The samples were combined with simulated salivary fluid (pH 7) containing amylase (300 U/mL) at a 1:2 ratio for two minutes at 37 °C. A 1:2 volume of simulated gastric juice (6 mL) (pH 3) containing pepsin (2000 U/mL) was then added and incubated at 37 °C for two hours. A 1:1 volume of simulated intestinal juice (9 mL) (pH 7) containing pancreatin (100 U trypsin activity/mL) and bile (10 mM) was then added and incubated at 37 °C for two hours. The entire digestive process was conducted with constant and gentle agitation. The gastric digestion was terminated by increasing the pH, and the total digestion was terminated by snap freezing at -20 °C. A control sample, containing only the digestive enzymes, was also prepared under the same conditions.

2.6. *E. coli* Inhibitory Activity of the Biochar Digesta

Overnight-grown *E. coli* pathotypes F4+ and F18+ were inoculated to reach an initial OD₆₀₀ of between 0.030 and 0.050 into tubes containing 15 mL of LB broth supplemented with 0, 150, and 300 µL/mL of biochar digesta. All tubes were incubated at 37 °C with agitation (180 rpm). Bacterial growth was assessed by measuring the optical density of each culture at 600 nm (OD₆₀₀) at one-hour intervals using a spectrophotometer (UV/VIS Lambda 365, PerkinElmer, Waltham, MA, USA). The growth of the *E. coli* was also monitored in tubes containing equivalent concentrations of only enzyme digestion. The analysis was performed in biological duplicates with three technical replicates ($n = 18$).

2.7. Growth Effects of Biochar Digesta on *L. plantarum* and *L. reuteri*

In order to reach an initial OD₆₀₀ of between 0.12 and 0.14, overnight-grown *Lactobacillus* sp. was inoculated into tubes containing 15 mL of MRS broth supplemented with 0, 150, and 300 µL/mL of biochar digesta. All tubes were incubated under microaerophilic conditions at 30 °C without agitation. Bacterial growth was assessed by measuring the optical density of each culture at 600 nm (OD₆₀₀) at one-hour intervals using a spectrophotometer (UV/VIS Lambda 365, PerkinElmer, Waltham, MA, USA). The growth of the *Lactobacillus* sp. was also monitored in tubes containing equivalent concentrations of only enzyme digestion. The analysis was performed in biological duplicates with three technical replicates ($n = 18$) [20].

2.8. Statistical Analysis

The adsorption capacity was statistically analyzed by one-way ANOVA, using GraphPad Prism 9 (2020). Significant differences ($p < 0.05$) between the mean values are indicated by superscript letters in the graph. The statistical analysis of the growth test was performed by analysis of variance (two-way ANOVA) using GraphPad Prism 9 (2020). Significant

differences between the mean values were determined by Tukey's test at a 95% confidence level. Superscript letters in the graph indicate $p < 0.05$.

3. Results

3.1. Morphological Analysis

The biochar samples showed a composition of small fragments that had generally lost their morphological characteristics, as occurred in biochar from different plant material [21]. Only a few fragments had retained the structure of plant tissues, particularly elements belonging to the xylem, such as fibers or vessels. Most of these fragments were likely part of the vascular bundles of the primary structure of the stem or other waste organs from vine pruning. Fragments of biochar ranged from 80 to 2 μm (Figure 1A,D) and showed both smooth and rough surfaces (Figure 1E, arrow and arrowhead, respectively). Only a few fragments appeared as tubules of bundled vessels (Figure 1B,C). Occasionally, large fragments derived from secondary xylem were observed, which showed vessels with pits, fibers, or pit rays (Figure 1F; arrows, arrowheads, and brackets, respectively). All these elements included tubes with areas ranging from 70 to 1.5 μm^2 , which were mostly represented by primary vessels and fibers (Figure 1C,F). The large vessels of the secondary xylem had a diameter of about 20 μm , whereas parenchymatic cells of the pit rays represented open spaces with an area of 40 μm^2 (Figure 1F).

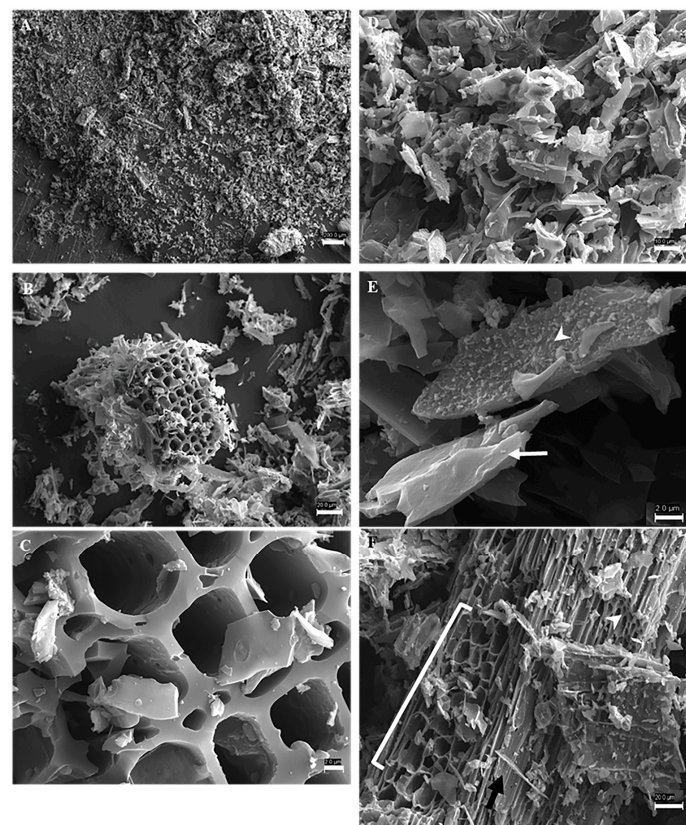


Figure 1. Morphological analysis of VB biochar by SEM. (A,D): Low magnification showed that the biochar appeared to be composed of small fragments. Only a few fragments retained the structure of plant tissues. (B,C) Higher magnification showed that some fragments presented elements belonging to the xylem, such as fibers or vessels. (E) Fragments of biochar showed both smooth and rough surfaces (E, arrow and arrowhead, respectively). (F) Occasionally, large fragments derived from secondary xylem were observed showing vessels with pits, fibers, or pit rays (F; arrow, arrowhead, and bracket, respectively). Magnification bars: (A) 200 μm ; (B,F) 20 μm ; (E,C) 2 μm ; (D): 10 μm .

3.2. *E. coli* Adsorption by Biochar

The effects of vine biochar on *E. coli* F4+ and F18+ adsorption were analyzed, and the results are shown in Figure 2. The number of *E. coli* colonies generally decreased after incubation with vine biochar. When *E. coli* F4+ was incubated with 0, 5, 10, and 20 mg of vine biochar at 37 °C for two hours, the total number of bacteria was $148.00 \pm 18.92 \times 10^6$, $78.83 \pm 7.51 \times 10^6$, $62.42 \pm 7.19 \times 10^6$, and $44.73 \pm 7.77 \times 10^6$ cells/mL, respectively. After two hours of contact, the percentage adsorption of *E. coli* F4+ was at 46.7%, 57.8%, and 69.8%, respectively (Figure 2A). On the other hand, when *E. coli* F18+ was incubated with 0, 5, 10, and 20 mg of vine biochar at 37 °C for two hours, the total numbers of bacteria were $157.00 \pm 7.20 \times 10^6$, $141.00 \pm 10.66 \times 10^6$, $86.25 \pm 7.78 \times 10^6$, and $68.92 \pm 10.27 \times 10^6$ cells/mL, respectively (Figure 2B). The percentage sorption of *E. coli* F18+ was at the respective concentrations of 10.2%, 45.0%, and 56.1% after two hours of contact (Figure 2B).

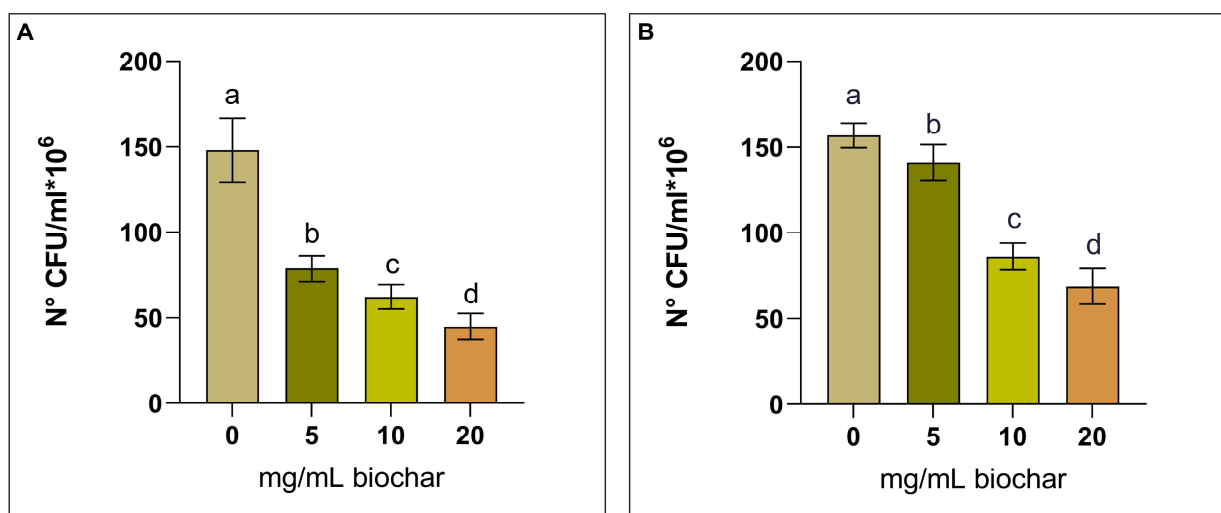


Figure 2. Adsorption of *E. coli* F4+ (A) and F18+ (B) with 0, 5, 10, and 20 mg/mL of VB biochar after two hours. The results are expressed as CFU/mL ± SD. Letters represent significant differences ($p \leq 0.05$).

3.3. *L. plantarum* and *L. reuteri* Adsorption by Biochar

The effects of vine biochar on *L. plantarum* and *L. reuteri* adsorption were analyzed, and the results are shown in Figure 3. In general, the numbers of *L. plantarum* decreased after incubation with VB biochar. When *L. plantarum* was incubated with 0, 5, 10, and 20 mg of VB biochar at 30 °C for two hours, the total number of bacteria was $150.30 \pm 2.53 \times 10^6$, $121.90 \pm 6.76 \times 10^6$, $101.20 \pm 6.50 \times 10^6$, and $92.17 \pm 5.78 \times 10^6$ cells/mL, respectively. The percentage adsorption of *L. plantarum* was at the respective biochar concentrations of 18.9%, 32.7%, and 38.7% after two hours of contact (Figure 3A). On the other hand, when *L. reuteri* was incubated with 0, 5, 10, and 20 mg of vine biochar at 37 °C for 2 h, the total number of bacteria was $193.80 \pm 8.94 \times 10^6$, $167.50 \pm 8.71 \times 10^6$, $146.60 \pm 5.65 \times 10^6$, and $122.9 \pm 9.55 \times 10^6$ cells/mL, respectively (Figure 3A). The percentage sorption of *L. reuteri* was at the respective concentrations of 13.6%, 24.3%, and 36.6% after two hours of contact (Figure 3B).

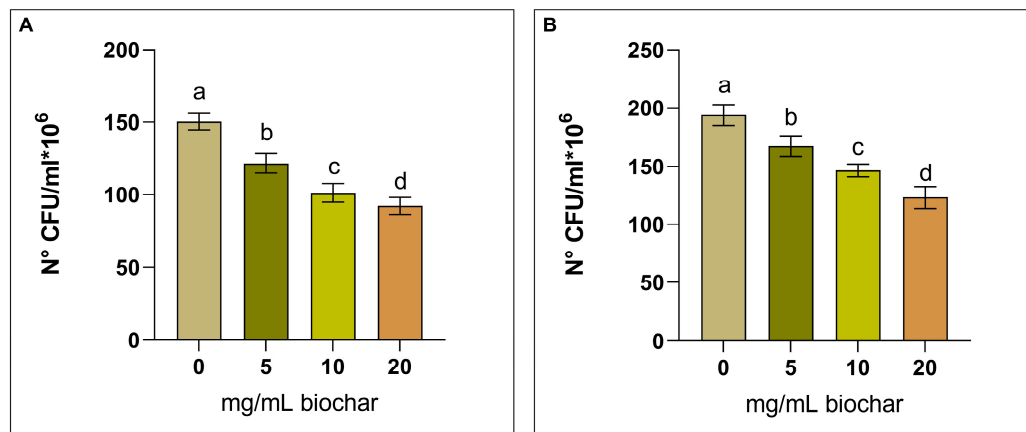


Figure 3. Adsorption of *L. plantarum* (A) and *L. reuteri* (B) with 0, 5, 10, and 20 mg/mL of VB biochar after two hours. The results are expressed as CFU/mL $\pm$ SD. Letters represent significant differences ($p \leq 0.05$).

3.4. Growth Inhibitory Activity Against *E. coli*

As shown in Figure 4, the growth of both *E. coli* pathotypes F18+ and F4+ was inhibited when exposed to biochar digesta fluid, and the inhibition generally started after three hours of contact. The growth inhibition of *E. coli* F18+ started at three hours of contact, with inhibition rates of 14% and 24% for 150 and 300 μ L/mL digesta, respectively. At five hours, the inhibition rates reached 26.8% and 35.4%, respectively, compared to those of the control (Figure 4A). The growth inhibitory activity for pathotypes F4+ occurred after three hours, with an inhibition rate of 29% for the maximum concentration tested, which remained similar after five hours (28%) (Figure 4B).

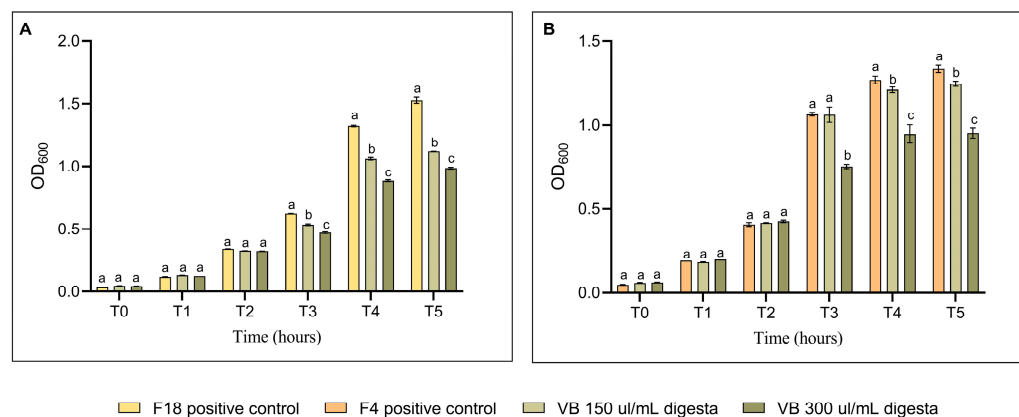


Figure 4. Growth inhibitory activity of biochar digesta against *E. coli* F18+ (A) and *E. coli* F4+ (B) at doses of 150 and 300 μ L/mL for five hours of contact. Different letters indicate significant differences at $p < 0.05$ among different concentrations within the same timepoint.

3.5. Probiotic Activity on *L. plantarum* and *L. reuteri*

In general, at both concentrations (150 and 300 μ L/mL) of biochar digesta, both *L. plantarum* and *L. reuteri* showed no inhibitory activity (Figure 5). The growth of *L. plantarum* after two hours of contact with biochar digesta was stimulated until the end of monitoring (eight hours) (Figure 5A). In contrast, the growth of *L. reuteri* was similar to the control during the first four hours of contact, and growth increased after six hours (Figure 5B).

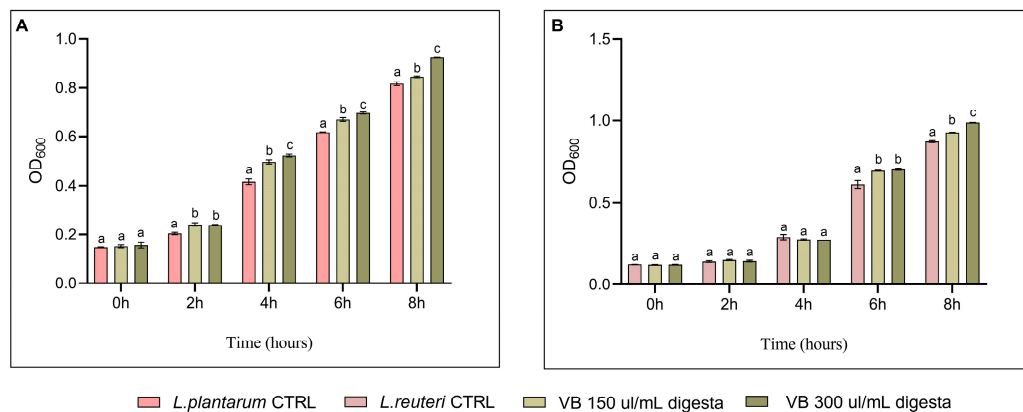


Figure 5. Probiotic activity of biochar on *L. plantarum* (A) and *L. reuteri* (B) at doses of 150 and 300 µL/mL for five hours of contact. Different letters indicate significant differences at $p < 0.05$ among different concentrations within the same timepoint.

4. Discussion

Given the growing interest in the use of biochar in different fields, this study evaluated how biochar interacts with both pathogens and probiotics and thus how it influences their growth, thus providing evidence supporting its use in animal nutrition.

One of the key elements is the sorption capacity, which we evaluated in biochar from vine pruning residue on pathogens and probiotic strains. Regarding pathogen microorganisms, we focused on *Escherichia coli*, especially the F4+ and F18+ strains. Since enterotoxigenic *Escherichia coli* (ETEC) characterized by F4 and F18 fimbriae are major causes of postweaning diarrhea in pigs and thus lead to significant economic losses, finding a functional ingredient that can help to prevent the development of pathologies is essential [22]. *Lactobacillus* sp., especially *L. reuteri* and *L. plantarum*, were chosen as probiotics since these strains are widely used in pigs and have demonstrated the potential to promote growth, improve feed use, and regulate the immune system in pigs [23].

The production of biochar at relatively low pyrolysis temperatures, such as those applied to vine pruning residues, promotes the development of a predominantly mesoporous structure, along with a rough and spongy surface and an overall amorphous carbon matrix. These morphological features are attributed to the partial preservation of the anatomy of the original plant tissue, particularly xylem elements such as fibers and vessels. These structures thus contribute to a hierarchical porosity, which can increase the adsorption capacity of biochar.

The irregular surface texture and tubule-like features derived from xylem cells can increase the surface area available for adsorption.

The microbial adsorption of biochar is a complex process governed by many chemical and physical factors. These interactions, which are not only linear, are strongly affected by the environmental conditions and context, thus making case-specific analyses necessary to fully understand their dynamics [24]. Our results revealed that the sorption activity of biochar on *E. coli* was generally strong, with *E. coli* F4+ exhibiting a higher percentage of sorption than *E. coli* F18+. At the maximum dose tested (10 mg/mL), biochar exhibited a percentage adsorption of *E. coli* F4+ of 69.8% in comparison to 56.1% for *E. coli* F18+. In addition, biochar has been observed to exhibit an adsorption capacity for *Lactobacillus* sp. At a concentration of 10 mg/mL, biochar has been found here to demonstrate an adsorption capacity of 38.7% and 36.6% for *L. plantarum* and *L. reuteri*, respectively.

The difference in the adsorption capacity of biochar revealed in this study between *E. coli* and *Lactobacillus* spp. could be due to the contrasting properties of these bacteria and the chemistry of the biochar. This depends on different factors, including the surface area,

pore structure, functional groups, and environmental conditions, which jointly influence the sorption mechanism. *E. coli* cells show a typical size of 0.5–2 µm, which, compared to that of *Lactobacillus* cells (approximately 2–4 µm), are better able to fill the pore size of biochar, thus increasing the selectivity towards this pathogen microorganism and demonstrating a greater adsorption efficacy [25]. In addition to the particle size effect previously discussed, another factor influencing the differential adsorption behavior is the surface chemistry of the biochar. A negative point of zero charge (ZPC) indicates that the biochar surface is predominantly negatively charged under neutral pH conditions, which may partially explain the observed adsorption of *Lactobacillus* sp. However, the cell wall of *Lactobacillus* is primarily composed of peptidoglycan containing positively charged surface regions, particularly amino groups ($-\text{NH}_3^+$), which facilitate electrostatic attraction with the negatively charged biochar surface [26].

Despite the overall negative charge of both the biochar and *E. coli* cells, significant adsorption of *E. coli* was still observed. This suggests the involvement of additional mechanisms beyond electrostatic interactions. For instance, carboxylate groups on the biochar surface can act as hydrogen bond acceptors and interact with hydrogen bond-donating functional groups on bacterial surfaces, such as amines ($-\text{NH}_2$) and hydroxyls ($-\text{OH}$) [27]. In addition, a biochar enriched in carboxylic groups tends to be hydrophilic, potentially leading to a higher affinity for hydrophilic bacteria such as *Escherichia coli* compared to the generally more hydrophobic *Lactobacillus* species [28]. The hydrophobicity of the *Lactobacillus* species shows considerable variability, with notable differences not only between species but also among strains within the same species. For example, *Lactobacillus plantarum*, as previously demonstrated, is more hydrophobic than *Lactobacillus reuteri* [23]. This difference is significant because compared to *L. reuteri*, *L. plantarum* is typically associated with a greater ability to adhere to hydrophobic surfaces, including mucosal tissues such as those found in the gastrointestinal tract [29,30]. This difference in adhesion capacity may reflect their distinct abilities to colonize various environments. The greater hydrophobicity of *L. plantarum* could contribute to its more efficient colonization of surfaces in the gastrointestinal tract, whereas *L. reuteri* may exhibit lower colonization efficiency due to its lower hydrophobic properties. The membrane structure of *L. reuteri* also generally contains fewer hydrophobic domains, which translates into a lower level of adhesion to surfaces such as VB biochar, which has hydrophobic characteristics [31]. These variations in membrane composition and surface interactions could help explain the different ecological niches occupied by these two species and their different functional roles in the microbiota.

The results obtained could have important implications for nutritional applications. The high sorption capacity to bind pathogenic *E. coli* suggests that orally administered biochar may be able to modulate the gut microbial population, thus minimizing the interference with normal and beneficial gut flora. In fact, Naka et al. (2000) [18] demonstrated the ability of activated charcoal to adsorb the *E. coli* O157:H7 strain in a dose-dependent manner and to absorb verotoxin 2 from the bacterial extract. Moreover, the activated charcoal did not demonstrate a strong affinity for *Enterococcus faecium*, *Bifidobacterium thermophilum*, and *Lactobacillus acidophilus*. The interaction between biochar and soil microorganisms has also been widely demonstrated since the addition of biochar promotes the growth of beneficial microorganisms [32].

Several studies have explored the use of biochar as a carrier for microorganisms due to its selective adsorption capacity and ability to create favorable environments for microbial growth. For instance, Zhang et al. (2022) [33] demonstrated that biochar combined with *Herbaspirillum huttiense* increases the remediation of heavy metals in contaminated soils. Similarly, Tu et al. (2015) [34] showed that maize straw biochar, when paired with *Pseudomonas frederiksbergensis*, improves plant growth and soil health in contaminated

environments. Saeed et al. (2023) [35] further supported this idea, highlighting the role of biochar in enhancing the activity of *Aeromonas hydrophila* for more efficient wastewater treatment. Biochar has also shown the potential to support various beneficial microbes, such as *Bacillus* spp. and *Rhizobium* spp., for bioremediation and plant growth promotion [36–38]. These studies underscore the versatility of biochar as a valuable tool in bioremediation and agricultural applications. By providing a stable surface for microbial attachment and improving microbial survival rates, biochar can significantly increase the effectiveness of microbial interventions in contaminated soils, wastewater treatment, and plant growth.

Evaluating the adsorption properties of biochar is important when considering biochar as a functional ingredient. However, the maintenance of functional properties following the digestive process also needs to be assessed. This study focuses on the antimicrobial and prebiotic properties since the main goal was to evaluate biochar as a functional ingredient that could promote the beneficial microorganisms in the gut microbiota of animals and reduce the occurrence of diseases.

Our results revealed that the in vitro digestion process could release functional compounds with modulating activity toward the microorganisms tested. Pathogens such as *E. coli* F4+ and F18+ were inhibited without a negative influence on probiotics, such as *Lactobacillus* sp. Using biochar as animal feed requires an in vitro study of simulated digestion in order to assess the potential interactions with the gut environment and ensure safety and efficacy [39]. In vitro studies are lacking on the effects of digested biochar on microorganisms. However, some in vitro studies have highlighted the functional properties of biochar, highlighting its antimicrobial and probiotic potential [21]. However, in vivo studies are also limited. Chu et al., (2013) [40] found a lower number of coliform bacteria in feces of pigs fed 0.3% of bamboo biochar, thus suggesting the possibility that biochar may have an effect on the proliferation of *E. coli*, which is the dominant microorganism responsible for inducing diarrhea in young pigs.

Our results highlight the dual role of biochar in reducing pathogens and supporting beneficial microbes, thus making biochar a potential functional ingredient in animal feed. *Lactobacillus* sp. or, in general, lactic acid bacteria (LAB) are used to prevent or treat intestinal infection or diarrhea caused by pathogenic bacteria through different mechanisms (e.g., stimulation of immunity, competition for nutrients, inhibition of epithelial and mucosal adherence, inhibition of epithelial invasion, and production of antimicrobial substances) [41]. The impact of probiotics can be augmented with the inclusion of biochar.

This synergy exploits the adsorptive properties of biochar, which create a more favorable gut environment, in conjunction with the direct benefits of probiotics in strengthening the gut microbiota and improving gastrointestinal health. On the other hand, *Escherichia coli* is a common pathogen in livestock farming, particularly in pig production, where it causes gastrointestinal diseases that impair animal growth and disrupt production cycles, leading to significant economic losses [22]. The results obtained in this study, although limited to the laboratory setting, highlight the potential of biochar as a functional ingredient, which could become a valuable addition to animal feedstuffs by helping to reduce bacterial infections. This, in turn, could reduce the need for antibiotics and alleviate the economic pressures caused by infectious diseases on livestock farms. However, the safety, efficacy, and practical operation of biochar need to be thoroughly evaluated in in vivo studies in order to verify its applicability in real-world settings.

5. Conclusions

In the context of sustainable development in the agro-livestock sector, assessing the uptake capacity of beneficial and/or pathogenic microorganisms is crucial for evaluating possible functional ingredients. This study highlights the ability of biochar during in vitro

digestion to adsorb and inhibit pathogens such as *E. coli*, which is the main cause of intestinal disease. In addition, its ability to promote the growth of probiotics in simulated digestion confirms biochar as a functional food. These findings provide a valuable basis for further research, particularly to explore the effects of biochar on a wider range of microorganisms and for an in vivo evaluation test to confirm the in vitro results.

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