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Reduction of *Escherichia coli* through *Lactobacillus casei* Fermentation in Simulated Fecal Matter

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Abstract

Introduction: The increasing population, water scarcity, and resource limitations challenge sanitation management, especially in developing countries. Traditional wastewater treatments, like flush toilets and chemically treated portable toilets, have drawbacks such as high energy consumption and odor issues. Lactic acid fermentation using *Lactobacillus* spp. presents a cost-effective, sustainable alternative by lowering pH and inhibiting pathogens. This study evaluated the effect of *Lactobacillus casei* fermentation on simulated fecal matter for potential use in fishing vessels, rural areas, and resource-limited settings.

Methods: *L. casei* exhibited exponential growth up to 8 hours before stabilizing, with reduced proliferation at ambient temperature. Simulated fecal matter was prepared with microcrystalline cellulose, psyllium husk, oleic acid, mineral salts, soybean flour, and 15% molasses to enhance lactic acid production. Experiments were conducted using simulated feces compared to human feces, evaluating pH, microbial concentration, and physicochemical parameters.

Results: Fermentation with *L. casei* (10% and 20%) reduced pH to approximately 4.0 within six days, remaining stable until day 30. Lactic acid production followed a similar trend, while *Escherichia coli* concentrations (initially 2.55×10^7 CFU/g) significantly decreased, with maximum reduction by day six. Comparable effectiveness between 10% and 20% *L. casei* suggests reduced inoculum concentrations may suffice for microbial control.

Conclusion: These findings support *L. casei* fermentation as a promising biotechnological approach for pathogen reduction in waste treatment. The potential application of this method in portable sanitation systems, especially in rural areas with limited resources, is highlighted, contributing to sustainable waste management and pathogen control.

Keywords: *Lactobacillus casei*, *Escherichia coli*, Lactic acid fermentation, Fecal matter
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Introduction

Population growth, limited access to water, and resource scarcity pose significant challenges to sanitation in developing countries, often resulting in inadequate wastewater management and the absence of proper sanitary facilities (1). Feces, which contain undigested fats, proteins, water, polysaccharides, and a high bacterial load, are typically treated as waste (1–3). This material harbors high concentrations of pathogens, including fecal coliforms and other bacteria detrimental to public health (4). Developing sustainable sanitation systems that are safe, efficient, and durable remains a major challenge.

Traditional sanitation methods, such as flush toilets, present multiple drawbacks, including complex infrastructure, skilled sewage management requirements, high energy consumption, and odor issues (5). Thus, synthetic microbial engineering offers a promising approach to enhance the breakdown of recalcitrant macromolecules in human feces, which native microbes struggle to degrade. Synthetic microbiomes are composed of carefully designed microorganisms optimized for synergistic performance under varied conditions (6,7). In portable toilets, fecal matter is typically treated with chemical solutions. Previous research has explored treatments using ammonia, yeast, and lime (8–10). However, ammonia treatment results in unpleasant odors and the regrowth of pathogens, while lime requires frequent replenishment (8,11). An alternative approach involves lactic acid fermentation using various *Lactobacillus* species, including *Lactobacillus casei*, *L. plantarum*, *L. acidophilus*, and *L. delbrueckii*, sourced from rice and cassava (12–14). Bira *et al.* (15) emphasized that proper fecal management is essential to prevent health

risks like disease transmission and water contamination, while also reducing environmental impacts such as nutrient loading, microbial pollution, and groundwater degradation. They noted that human excreta can serve as a valuable organic fertilizer, enhancing soil properties like water retention, but require prior treatment (e.g., composting, anaerobic digestion, vermicomposting) to remove pathogens. Despite these benefits, in many low-income countries such as Ethiopia, social acceptance of using excreta remains low.

Lactic acid (LA) ($CH_3CH(OH)COOH$) is a naturally occurring hydroxycarboxylic acid that exists in two optically active isomeric forms, L and D, each imparting distinct physical properties to the final product (16). LA fermentation has gained interest due to its low environmental impact, inexpensive substrate requirements (e.g., food waste), moderate operating temperatures, and low energy consumption (17).

Lactic acid fermentation of fecal material is an effective strategy for odor control and pathogen reduction (4,18). By lowering the pH through acid production, this process inhibits the growth of pathogenic microorganisms, offering a novel and practical approach to fecal sanitation before further treatment (19). Additionally, it is a cost-effective and straightforward method that aligns with circular economy principles, making it an increasingly attractive solution in sustainable waste management (20,21). However, the large-scale implementation of lactic acid fermentation faces economic and environmental challenges, as recovery and purification processes can account for 20–50% of total operating costs, limiting its feasibility for widespread adoption (22). Further research is needed to optimize these processes and improve scalability.

This study aimed to assess the sanitizing effects of *Lactobacillus casei* fermentation in simulated feces as a potential alternative for portable sanitation systems, with applications in fishing vessels, rural areas, and other resource-limited settings. Despite progress in ecological sanitation, the specific role of *L. casei* in reducing *E. coli* through fecal matter acidification remains poorly studied. The combined effects of pH reduction, lactic acid accumulation, and potential bacteriocin production have not been explored in detail in simulated feces. This study addressed that gap by evaluating both biochemical changes and microbial dynamics.

Material and Methods

Activation of microbial strains

Lactobacillus casei ATCC 393 and *Escherichia coli* ATCC 25922 strains were used in this study. Both strains were obtained from Kwik Stik, Microbiologics. The *E. coli* ATCC strain was activated in MacConkey agar and nutrient broth, while *L. casei* ATCC was activated in Man Rogosa Sharpe (MRS) agar. Incubation was performed at 37°C for 18-24 hours for *E. coli* and 72 hours for *L. casei*. Following incubation, both macroscopic and microscopic characterization of the strains was conducted.

Growth kinetics of Lactobacillus casei ATCC 393

A pre-culture of *L. casei* was prepared in buffered peptone water and incubated overnight at 37°C. Absorbance at 600 nm was measured using a UV-Vis spectrophotometer. The growth of *L. casei* was then inoculated into 250 mL of peptone water at a known concentration and evaluated at three different temperatures (ambient: 22°C, 37°C, and 42°C) (23,24). Bacterial growth was measured at 0, 1, 3, 6, 9, and 24 hours on the first day, and subsequently every 24 hours for the following 5 days. Peptone water was used as the blank.

Experimental set-up

Preparation of simulated feces

The composition proposed by Penn *et al.* (2018) (25) with minor modifications, was selected as the most appropriate for this study. The mixture was designed to reproduce the main chemical and physical characteristics of human feces, following recommendations from various authors (25–29) and was adapted to laboratory conditions for experimental purposes related to in situ sanitation systems.

The approximate composition of natural human feces and the physiological role of each component are summarized in Table 1 (28). To reproduce this composition, different materials were selected: *Escherichia coli* inoculum to represent the bacterial biomass, microcrystalline cellulose and psyllium for the carbohydrate and fiber fraction (with psyllium additionally providing gelling), soy flour as a source of nitrogen and protein, oleic acid for the lipid fraction, and NaCl and KCl as mineral components (Table 2).

For preparation (Figure 1), the dry ingredients were first mixed in a container. Oleic acid was then incorporated and homogenized, followed by the gradual addition of the *E. coli* inoculum. The prepared mixture was then inoculated with previously activated *E. coli* (1.5×10^6 – 1.5×10^8 CFU/mL). The mixture was covered and left to rest for 90 min at room temperature to allow complete psyllium gelation (13). The final formulation corresponds to a standardized 100 g preparation of simulated feces.

Lactic acid fermentation in simulated faecal matter

An inoculum of *L. casei* was prepared at room temperature, reaching a concentration of 2.0 on the McFarland scale (6.0×10^8 CFU/mL). Four experimental groups were established to assess the effect of increasing concentrations of *L. casei* on the fermentation process. The control group contained no *L. casei*, while Groups 1, 2, and 3 were supplemented with 5%, 10%, and 20% (p/p) *L. casei*

Table 1. Approximate composition of natural human feces (% dry weight).

Components	Amount
Lipids	5-25%
Carbohydrates (Fiber)	10-30%
Nitrogenous material	≤2-3%
Minerals (mainly K, Ca y P)	5-8%
Bacterial waste	10-30%

Table 2. Chemical composition of simulated feces (100 g preparation).

Compound	Amount (g)	Function
Escherichia coli inoculum	30	Bacterial debris
Microcrystalline cellulose	10	Carbohydrate
Psyllium	17.5	Fiber fraction
Soy flour	17.5	Nitrogenous material and protein source
Oleic acid	20	Lipid fraction
NaCl	2.5	Mineral fraction
KCl	2.5	Mineral fraction

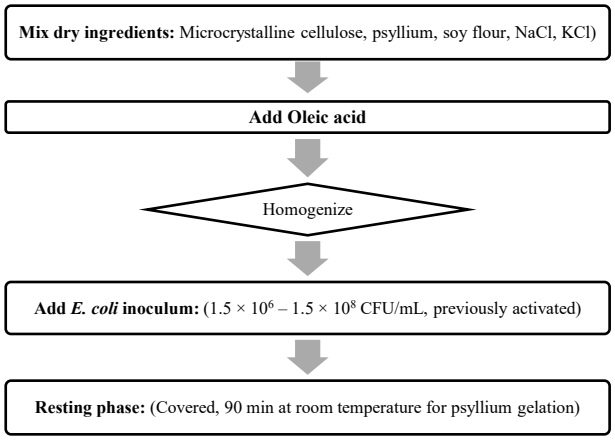

Figure 1. Step-by-step preparation of simulated feces under laboratory conditions

inoculum, respectively, with molasses concentration kept constant at 15% (Table 3). Fermentation was conducted in hermetically sealed containers and maintained at room temperature. pH and lactic acid concentration were measured daily, while *E. coli* and *L. casei* counts were performed and recorded every 3 days during the first 10 days. These parameters were re-evaluated on days 15 and 30.

pH determination

Approximately 1 g of the sample was suspended in distilled water to a final volume of 100 mL and agitated at 250 rpm for 10 minutes. The pH was measured using a model Starter 3100M pH benchtop meter (OHAUS Corporation, Parsippany, NJ, USA).

Determination of lactic acid in the fermentation process

The sample was prepared following the same procedure as for pH determination. Two drops of 1% phenolphthalein were added, and the mixture was titrated with 0.1 N NaOH, previously standardized with potassium acid phthalate, until a persistent currant-pink color appeared. Acidity was calculated as a percentage of lactic acid, considering that 1 mL of 0.1 N NaOH corresponds to 0.09 g of lactic acid, as described in Eq. 1. This analysis was conducted using the acid-base titration technique. Lactic acid concentrations were determined in triplicate, and the data were analyzed using one-way ANOVA followed by Tukey's post-hoc test, with significance level set at $P < 0.05$.

$$\%Acidity\ of\ lactic\ acid = \frac{G \times N \times F}{m} \times 100 \quad (1)$$

Table 3. Groups for the fermentation of lactic acid

Component (% p/p)	Control	Group 1	Group 2	Group 3
L. casei	0	5	10	20
Molasses	15	15	15	15
Fecal material with *E. coli*	65	65	65	65
Sterile distilled water	20	15	10	0
Final weight	100	100	100	100

Where G is NaOH expenditure (mL), N is NaOH normality, m is diluted sample (g), and F is lactic acid conversion factor (0.09).

Microbiological count of E. coli and L. casei

For *E. coli*, approximately 1 g of the sample was weighed and mixed with sterile distilled water to a final volume of 100 mL, then shaken at 250 rpm for 10 minutes. After appropriate dilutions, 1000 µL of the sample was inoculated onto MC-Media Pad EC medium and incubated at 37°C for 24 hours. The bacterial count was determined using the plate count method and expressed as CFU/g.

For *Lactobacillus casei*, approximately 1 g of the sample was mixed with peptone-buffered water to a final volume of 100 mL and agitated at 250 rpm for 10 minutes. After serial dilutions, 10 µL of the sample was spread onto the surface of an MRS agar plate using the spread plate technique with a Digrasky loop. The plates were incubated at 37°C for 48 hours, and the bacterial count was determined using the plate count method, expressed as CFU/g.

Results

Activation of microbial strains

The macroscopic and microscopic characterization of the microorganisms revealed distinct features. On MacConkey agar, *Escherichia coli* colonies appeared pink after 24 hours of incubation at 37°C, indicating lactose fermentation. In contrast, on nutrient agar, *E. coli* colonies were whitish, reflecting its non-lactose-fermenting nature on this medium. *Lactobacillus casei*, cultured on MRS agar, formed small, circular, whitish colonies, measuring 1-2 mm in diameter, with slow growth observed over a 48- to 72-hour period.

Microscopic examination through Gram staining revealed additional distinguishing characteristics. *E. coli* was observed as pink bacilli, consistent with its Gram-negative classification and non-spore-forming nature. In contrast, *Lactobacillus casei* stained violet, identifying it as a Gram-positive, rod-shaped bacterium. These observations provide clear macroscopic and microscopic profiles that aid in the precise identification and differentiation of *E. coli* and *L. casei*.

Growth kinetics of L. casei ATCC393

Figure 2 presents the growth curve of *Lactobacillus casei* on a logarithmic scale. The data reveal a distinct exponential growth phase from 0 to approximately 8 hours, followed by a stationary phase where bacterial growth stabilizes. Growth patterns were comparable at 37 and 42°C, exhibiting similar

trends in both time and bacterial concentration. However, at room temperature (22°C), a lower growth rate was observed, with a delayed exponential phase and a reduced overall bacterial concentration. These results suggest that while *L. casei* can proliferate across different temperatures, optimal growth occurs at 37 and 42°C, with diminished growth at lower temperatures.

Simulated fecal matter

To prepare the simulated feces, microcrystalline cellulose was utilized as a source of fiber and carbohydrates, while psyllium husk provided dietary fiber, carbohydrates, and water retention properties. Oleic acid was included to represent lipid content, and NaCl and KCl were added to supply essential minerals. Soybean meal served as the protein source. Furthermore, 15% molasses was added as a substrate, which facilitated the production of lactic acid. The physicochemical composition of the simulated feces was compared with reported parameters of human feces (Penn *et al.*, 2018) to validate its representativeness (Table 4). The pH and microbial load were consistent with expected ranges, although simulated feces lack the full microbial diversity present in human feces.

Determination of pH and Lactic acid

Figure 3 depicts the pH dynamics of the control and experimental groups over 30 days. Initially, all groups exhibited pH values ranging from 6.7 to 7.0. The control group maintained a stable pH throughout the study, with only minimal variations. In contrast, the experimental groups experienced a marked decline in pH during the first six days, after which it stabilized.

Groups 2 and 3, which contained 10% and 20% *Lactobacillus casei*, respectively, exhibited the most pronounced pH reduction, reaching approximately 4.0 by day 5 and maintaining this level until day 30. Meanwhile, Group 1 showed a moderate decrease, reaching a final pH of approximately 5.10. These findings suggest that the presence of *Lactobacillus casei* significantly influenced acidification, with higher concentrations leading to greater pH reductions.

Lactic acid concentration was monitored throughout the experiment (Figure 4), showing a progressive increase in the experimental groups. The highest levels were recorded from day 6 onward, surpassing 3%, and remained stable until day 30. Groups 2 and 3 exhibited similar trends in both lactic acid production and pH reduction. In these groups, lactic acid concentrations exceeded 3% by day 5 and continued to rise slightly, reaching final values of 3.76% and 3.82% on day 30, respectively. In contrast, Group 1 displayed a steady increase, reaching 2.62% by the end of the study. The control group showed minimal variations, maintaining lactic acid concentrations below 0.40% throughout the experiment.

The analysis of variance (ANOVA) revealed significant differences in lactic acid production among the treatment groups ($F(2,36) = 5.41$, $P = 0.009$). Post-hoc comparisons using Tukey's test in Table 5 indicated that Groups 2 (10% *L. casei*) and 3 (20% *L. casei*) produced significantly higher amounts of lactic acid compared to Group 1 (5% *L. casei*) ($P < 0.05$). However, no significant differences were observed between Groups 2 and 3, suggesting that increasing the inoculum concentration beyond 10% did not lead to further increases in lactic acid production.

Microbiological count

Figure 5 illustrates the *E. coli* concentration over 30 days. On day 0, the average *E. coli* concentration was 2.55×10^7 CFU/g. While the control group exhibited exponential growth, Groups 1, 2, and 3 showed a gradual decline in *E. coli* concentrations, reaching their lowest levels by day 6. By day 30, *E. coli* was undetectable in these groups. In contrast, the control group continued to experience an increase in *E.*

Table 4. Comparison of physicochemical and microbiological parameters between human feces and simulated feces.

Parameter	Human Feces (Penn et al., 2018)	Simulated Feces (This Study)
pH	6.6 – 7.0	6.7 – 7.0
Escherichia coli load	10^6 – 10^8 CFU/g	2.55×10^7 CFU/g

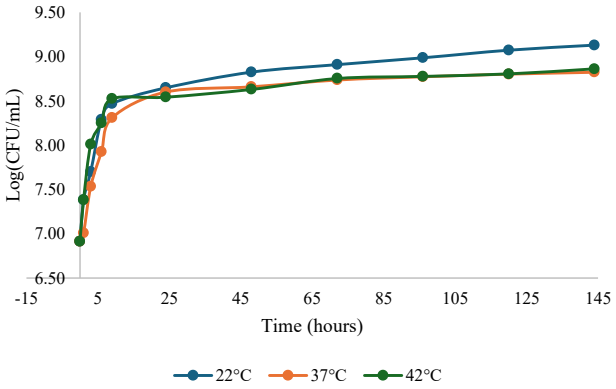

Figure 2. Bacterial growth curve of Lactobacillus casei ATCC 393 at different temperatures

Table 5. Grouping Information Using Tukey's test and 95% Confidence.

Group	N	Mean lactic acid (%)	Grouping
Group 3 (20%)	13	2.577	A
Group 2 (10%)	13	2.448	A
Group 1 (5%)	13	1.511	B

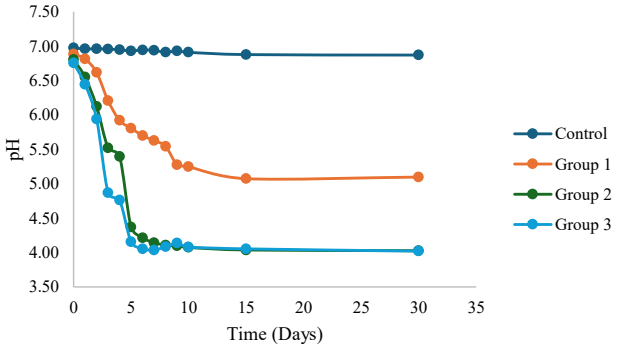

Figure 3. pH measurement obtained from fermentation in the study groups using different concentrations of *L. casei* for 30 days

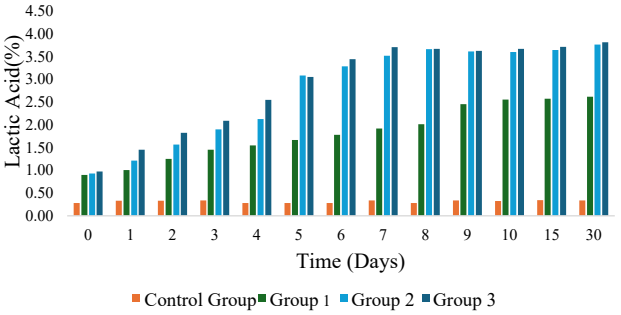

Figure 4. Lactic acid concentration (%) obtained from fermentation using different *L. casei* concentrations over 30 days

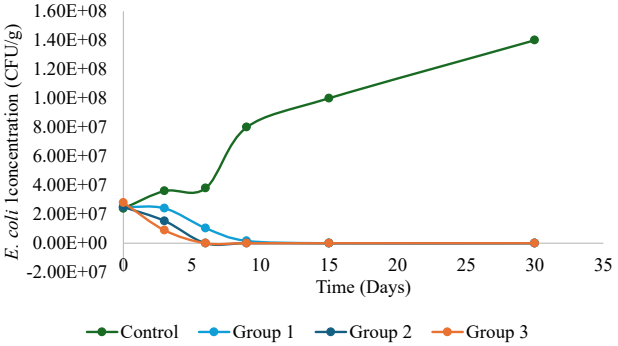

Figure 5. Growth behaviour of *E. coli* during lactic acid fermentation in simulated faecal matter over 30 days

coli concentration, reaching 1.40×10^8 CFU/g.

Figure S1 illustrates the growth of *L. casei* over 30 days. The most pronounced increase in *L. casei* concentration occurred on day 6, coinciding with the peak decline of *E. coli*. Following this peak, *L. casei* growth slightly decreased before stabilizing. On day 6, Group 3 exhibited the highest *L. casei* concentration, reaching 1.48×10^{10} CFU/g, while the control group and Group 1 showed lower values. In the subsequent days, *L. casei* concentrations experienced a slight decline before stabilizing by day 30. These findings suggest a potential relationship between *L. casei* growth and *E. coli* inhibition, indicating that *L. casei* may play a role in suppressing *E. coli* proliferation during the experimental period.

Discussion

The findings of this study highlight the distinct characteristics and growth patterns of *Lactobacillus casei* and *Escherichia coli* under controlled experimental conditions. Macroscopic and microscopic observations confirmed the expected phenotypic traits, facilitating their accurate identification and differentiation. Lactic acid fermentation has been reported to be influenced by the growth phase of *L. casei* (30).

The growth kinetics of *L. casei* ATCC 393 revealed an exponential phase lasting up to 8 hours, followed by a stationary phase (Figure 2). Similar growth patterns were observed at 37°C and 42°C, suggesting that these temperatures provide optimal conditions for *L. casei* proliferation. However, a significantly lower growth rate was noted at room temperature, emphasizing the crucial role of temperature in bacterial metabolism. The selection of room temperature for the fermentation process was supported by previous studies identifying this condition as favorable for *L. casei* preculture development (25). These findings align with those of Anderson et al. (8), who observed similar growth trends in milk cultures. Conversely, Acedos *et al.* (31) reported optimal growth at higher temperatures (50–55°C) in anaerobic digesters, highlighting the influence of environmental conditions on bacterial proliferation.

The formulation of simulated fecal matter successfully replicated the consistency of human feces. The inclusion of molasses as a substrate was justified by its ability to supply essential mineral salts and nitrogenous compounds, enhancing lactic acid production (25). The presence of *E. coli* in the simulated feces was confirmed with an initial concentration of 2.5×10^7 CFU/g, aligning with expected values for indicator bacteria. The consistency of the prepared material corresponded to Bristol Type 3, described as a sausage-like texture with surface cracks, which is considered normal (32,33).

The pH evolution observed in the fermentation groups underscores the role of *L. casei* in acidification. A significant pH reduction was observed in Groups 2 and 3, reaching values close to 4.0 by day 6 (Figure 3), suggesting a direct correlation between *L. casei* concentration and acid production. This trend is consistent with previous research reporting pH values below 5.0 within similar fermentation periods (4). The stabilization of pH after day 6 indicates that acid production reached equilibrium, a phenomenon commonly associated with the depletion of fermentable substrates.

The statistical analysis confirmed that lactic acid production differed significantly between groups ($P = 0.009$), suggesting that the inoculum concentration of *L. casei* plays a key role in the acidification process. This finding aligns with the findings of previous studies reporting that higher inoculum levels accelerate pH reduction and pathogen inhibition.

Lactic acid concentration trends (Figure 4) further support these pH changes, as acid production stabilized after day 6, aligning with the peak fermentation efficiency of *L. casei*. Previous studies using sugarcane molasses as a fermentation substrate have reported similar acidification kinetics, reinforcing the efficacy of this approach for lactic acid production (9). Masís-Meléndez et al. (9) confirmed that lactic acid production typically reaches a steady state within 10–30 days under these conditions, while Yemaneh *et al.* (18) demonstrated a significant reduction in *E. coli* concentrations to undetectable levels after 21 days when using 5–10 % molasses. These findings further validate the potential of sugarcane molasses as a reliable substrate for controlled lactic acid fermentation. Few studies have explored synthetic microbiomes for aerobic composting of human feces. Lactic acid fermentation using inoculants like sauerkraut juice can produce agriculturally valuable compost (7,34) but requires ~86 days, indicating low efficiency. This study highlights the

potential of *Lactobacillus casei* to improve treatment speed and effectiveness.

These findings are in line with the broader literature on fecal management. Bira et al. emphasized that adequate treatment of human excreta is crucial not only for pathogen reduction but also for minimizing environmental risks such as groundwater contamination and nutrient overload. Integrating lactic acid fermentation into fecal treatment could complement existing strategies (composting, anaerobic digestion, vermicomposting) by enhancing pathogen inactivation while maintaining the potential for agricultural reuse of sanitized excreta (15).

Microbiological analysis highlights the inhibitory effect of lactic acid fermentation on *E. coli* proliferation. While the control group exhibited continuous growth, Groups 1, 2, and 3 showed a marked decline in *E. coli* counts, reaching their lowest levels by day 6 (Figure 5). These findings align with those of previous reports where lactic acid fermentation significantly reduced pathogen levels within 10 to 30 days (2,4,18). Notably, the accelerated decline observed in this study, achieving substantial reductions within the first week, suggests that higher concentrations of *L. casei* enhance antimicrobial activity. Additionally, Andreev *et al.* (12) found that combining lactic acid fermentation with thermophilic composting effectively eliminated *E. coli* in human waste samples.

Finally, the growth dynamics of *L. casei* (Figure Supplementary File 1) peaked on day 6, coinciding with the maximum suppression of *E. coli*, suggesting a competitive exclusion mechanism potentially driven by acidification and bacteriocin production. The subsequent stabilization of *L. casei* indicates its ability to remain viable within the simulated fecal environment, contributing to the establishment of a balanced microbial ecosystem. These findings highlight *L. casei* as a promising candidate for biotechnological applications in waste treatment and pathogen control. Different studies have used varying concentrations of *L. casei* for lactic acid production, reporting fermentation periods ranging from 10 to 30 days (2,9). Our results are consistent with those of Odey *et al.* (13), who reported pH reduction to < 5.0 after lactic acid fermentation. Similarly, Masís-Meléndez *et al.* (9) observed complete inactivation of fecal coliforms after 21 days. However, our study achieved similar results in only 6 days, highlighting the efficiency of *L. casei*.

Overall, this study confirms the effectiveness of *L. casei* in reducing *E. coli* concentrations through lactic acid fermentation. These findings contribute to the understanding of probiotic applications in microbial control and support the potential use of *L. casei* for pathogen inhibition in various biotechnological and food safety contexts (2,4,9,18,25).

Conclusion

This study demonstrates the effectiveness of *Lactobacillus casei* in reducing *Escherichia coli* concentrations through lactic acid fermentation, highlighting its potential for microbial control and biotechnological applications. The fermentation process led to a significant pH reduction to approximately 4.0 and a lactic acid concentration exceeding 3.0%, effectively inhibiting *E. coli* proliferation. Notably, no significant differences were observed between the 10% and 20% *L. casei* groups, suggesting that lower inoculum concentrations may be sufficient for microbial suppression.

The metabolic activity of *L. casei* likely contributes to *E. coli* inhibition through acidification and potential bacteriocin production, creating an inhospitable environment for pathogen survival. These findings support the use of *L. casei* fermentation as a cost-effective and sustainable approach for pathogen control, particularly in waste treatment and sanitation management. Given its efficacy in simulated fecal environments, this method represents a promising pre-treatment strategy for portable toilets, especially in resource-limited settings such as fishing boats and rural areas.

Moreover, the ability of *L. casei* to maintain viability throughout the fermentation process suggests its potential role in establishing a stable microbial ecosystem that may further enhance waste degradation. Future research should focus on the long-term stability, scalability, and broader environmental implications of *L. casei* fermentation to optimize its application as a biocontrol agent in industrial and public health contexts. While the results in simulated feces demonstrate strong hygienization potential, future validation with real human feces is necessary to confirm the generalizability of these findings.

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Authors' Contribution

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Methodology: Yohiss del Rocío Medina-Chávez, Daniela Camacho-Valencia, Stamber Alvaro Ramírez- Revilla.
Project administration: S.A Ramírez-Revilla.
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Writing - review & editing: Yohiss del Rocío Medina- Chávez, DanielaCamacho-Valencia, José Antonio Villanueva-Salas, Stamber Alvaro Ramírez-Revilla.

Competing Interests

There are no conflicts of interest associated with this publication.

Ethical Approval

This study did not involve human or animal subjects; therefore, no ethical approval was required.

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Supplementary Files

Supplementary file 1 contains Figure S1.

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