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Improving the Quality and Safety of Barley by Controlled Homolactic Fermentation with Lactic Acid Bacteria

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Abstract

Fermentation by lactic acid bacteria can improve the nutritional and hygienic quality of barley, as well as its organoleptic properties. The aim of this study was to select a suitable lactic ferment to add value to barley through a controlled homolactic fermentation process, which can be used to produce a natural base product for broiler feed. Four different combinations of lactic strains were tested for their acidifying power, antibacterial activity, and fermentation performanceThe mixed culture of homolactic ferment FS4, composed of *Lactobacillus plantarum* (SC1), *Pediococcus spp* (SC4), and *Lactococcus lactis ssp* (SC5), showed the best result in terms of pH reduction, acidity production, and inhibition of spoilage bacteria. The in *vivo* application of the ferment FS4 on barley resulted in a decrease in pH to 4.12 after 15 days of fermentation, and an improvement in its hygienic quality, with the possibility of also enhancing its nutritional quality. This study demonstrated that FS4 is a suitable lactic ferment for valorizing barley through a controlled homolactic fermentation process, which can be used to produce a natural base product for animal feed with improved quality and safety.

Keywords: Barley, Fermentation process, Lactic ferment, Valorizing

Introduction

Fermentation is a natural phenomenon that occurs during the decomposition of organic matter. It is also one of the most widely used methods for preserving and processing food raw materials [1]. Barley, *Hordeum vulgare*, is a cereal with high energy content but low protein content, which limits its use in animal feed [2]. One of the main challenges in using barley as feed is the presence of β-glucans, which are considered an antinutritional factor that reduces digestibility and increases viscosity in the gastro-intestinal tract. Therefore, there is a need to find ways to improve the nutritional and hygienic quality of barley as feed.

Fermentation by lactic acid bacteria (LAB) is a potential method to enhance the quality and safety of barley as feed. LAB are microorganisms that secrete lactic acid and

other substances that inhibit the growth of pathogens, spoilage bacteria and fungi [3,4]. They also provide desirable organoleptic characteristics and properties to fermented foods [5]. Moreover, they can produce bacteriocins, which are small peptides with antimicrobial activity [6].

Lactic acid bacteria possess the ability to decompose and convert components like starch, cellulose, hemicellulose, and lignin found in biomass raw materials. Furthermore, they enhance material reactivity and establish interactions with their metabolites and sugars, effectively contributing to the progression of fermentation [7]. In this context, LAB of the homolactic fermentative type are previously inoculated into silage, as they have the ability to dominate fermentation by growing rapidly and producing a higher quality product [8]. Several studies have reported the benefits of using LAB fermentation on cereals such as wheat, maize, sorghum, and rice [9]. However, there is limited research on the effects of LAB fermentation on barley as feed.

The aim of this study was to select a suitable lactic ferment to add value to barley through a controlled homolactic fermentation process. This process can produce a natural base product for broiler feed. The characteristics of the chosen fermenter were meticulously matched with the specific requirements of the barley fermentation procedure. This ensured optimum conditions for the desired results. This systematic approach emphasizes the importance of fermenter selection and facilitates the accuracy and efficiency of the controlled fermentation process for local barley. Four different combinations of lactic strains were tested for their acidifying power, antibacterial activity, and fermentation performance. The selected ferment was then applied on barley in *vivo* to evaluate its quality and safety. This study contributes to the development of novel fermented products from barley that can improve its nutritive and hygienic value as feed.

Materials and Methods

Selection Criteria for Lactic Acid Strains

In order to determine the most suitable combination of LAB for ferment preparation, the choice of strains in pure culture was based on five criteria: homolactic fermentation type; ability to grow at non-optimal temperatures; powerful acidifying effect, which allows adaptation to different environmental conditions; strong antibacterial activity, which prevents the contamination of pathogens; and compatibility with other strains, which enables synergistic interactions.

Ferment Selection

Composition of proposed lactic ferments

Three lactic strains, *Lactobacillus plantarum* (SC1), *Pediococcus spp* (SC4) and *Lactococcus lactis ssp* (SC5), were used in this study. These strains were previously isolated from three preparations of fermented barley and identified by API 50CHL Medium gallery (Bio-Merieux reference 50410) [10]. The various combinations of strains were added to liquid MRS culture media in different proportions, as shown in Table 1. The solutions were incubated at 30°C for 48 h.

Table 1. **Different combinations of lactic strains in fermented solutions.**

Ferment code	Solution
FS1	*Lactobacillus plantarum +Pediococcus spp*
FS2	*Lactobacillus plantarum + Lactococcus lactis ssp*
FS3	*Pediococcus spp + Lactococcus lactis ssp*
FS4	*Lactobacillus plantarum + Pediococcus spp + Lactococcus lactis ssp*

Characteristics required for ferment screening

Ferment selection was based on the compatibility of its constituent strains, and the following characteristics: (i) acidifying power, determined by measuring pH and acidity expressed as a percentage of lactic acid, and (ii) antibacterial activity, determined by the well diffusion method on Mueller-Hinton agar (MHA); after elimination of the effect of organic acids by neutralization (NaOH 0.1 N) and elimination of the effect of oxygen peroxide by addition of a few drops of catalase to the cell-free culture supernatant. Petri dishes were first stored at 4 °C for 4 h to allow diffusion of the supernatant into the agar and then incubated at 35±2 °C for 24 h. The spoilage strains tested were previously isolated and identified in our laboratory [10]. The solution with the desired properties was the right combination for our fermentation.

Ferment preparation and application trial

Ferment preparation involved determining the amount of starter needed to start controlled fermentation correctly. For this purpose, 3ml cultures were prepared from each strain in liquid MRS culture media. Cascade cultures were then made until the final volume of 1 L was obtained. All incubations were carried out at 30°C for 24 h. Before starting fermentation, the barley gains were broken in an electric grain mill to facilitate the process.

Ferment application involved inoculating the barley grains with the prepared ferment. For this purpose, the barley grains were broken in an electric grain mill to facilitate the process, and the broken grains were mixed with the fermented solution. The mixture was then incubated at ambient temperature in the dark. The fermentation time was determined by the ferment.

Parameters measured during fermentation

During the fermentation trial, a control follow-up of this process is carried out. First of all, at each sampling, the product's odor is checked to verify the presence or absence of unpleasant odors. The physicochemical parameters tested include pH measurement using a combination electrode pH meter, temperature measurement using a mercury thermometer, and moisture determination by steaming at 130°C for 2 h, expressed as a percentage (%), from which the percentage of dry matter is calculated by the difference in weight between pre-drying and post-drying.

Fermentation control monitoring was complemented by microbiological analyses to determine the microbial load contained in the product. The following germs were counted during fermentation:

- Flora of hygienic interest, which includes total aerobic mesophilic flora (TAMF). The count of total germs provides information on the overall bacterial load of the sample. Seeding was carried out on Plat Count Agar (PCA) medium.Cultures were incubated at 30°C for 72 h [11];
- Total coliforms (TC) were counted on Methylene Blue Eosin (EMB) agar after incubation at 37 °C for 24 to 48 h. Fecal coliforms (FC) were incubated at 44°C for 24 h and enumerated on MacConkey medium [12];
- Fungal flora (molds and yeasts), were counted on Potato dextrose agar (PDA) medium after incubation at 25°C for 72 h [13];
- Flora of biotechnological interest (LABa), counted on de Man Rogosa and Scharpe (MRS) agar medium after incubation at 30°C for 48 h [14].

The results were expressed in colony forming units (CFU) per gram.

Statistical analysis

Each test was repeated three times at different times to minimize experimental error. Data were expressed as mean values ± standard deviation, analyzed using SPSS software (V.23). Significant differences between pH, acidity and antimicrobial activity measurements were determined by a one-way analysis of variance (ANOVA); pH and acidity were significant at the level of $P < 0.01$, while antimicrobial activity is significant at $P < 0.05$. Significant differences between the means of microbiological monitoring of CT, Fungal Flora and LAB during fermentation were determined by a one-way ANOVA, followed by Tukey (post-hoc) test at a significant threshold of $P \leq 0.05$.

Results and Discussion

Ferment Selection

Initially, strains (SC1, SC4 and SC5) showed a homolactic type of fermentation [15]. Indeed, the metabolic pathway of homo-fermentative LAB leads to the production of 90% lactic acid and only 10% CO_2 [16]. In addition, these strains also showed an ability to grow at non-optimal temperatures (18, 20 and 22°C) and a pH ranging from 4 to 6, and exhibited significant acidifying power and strong inhibitory potential in pure culture against five spoilage strains, isolated from chicken meat and identified as; *Salmonella sp, Proteus vulgaris, Escherichia coli, Staphylococcus aureus* and *Klebsiella pneumonia* [10]. Thus, for each proposed combination, pH and acidity were monitored. The results in Table 2 showed that pH decreased after 48 h of incubation for all ferments. This indicates that the ferment combinations were able to lower the pH and increase the acidity of the medium, which are desirable characteristics for fermentation quality and safety.

Table 2. **pH and acidity values of different ferments grown in liquid MRS medium for 48 h at 30 °C.**

Ferment code	Mixed culture	pH	Acidity (% of lactic acid)
FS1	SC1+ SC4	3.46±0.03	1.68±0.05
FS2	SC1+ SC5	3.46±0.15	1.66±0.23
FS3	SC4+ SC5	3.48±0.24	1.65±0.33
FS4	SC1+ SC4+ SC5	3.40±0.22	1.71±0.35

On the basis of the results obtained and statistical analysis, we note that if pH is low, acidifying power will be high, which indicates the ability of LAB to produce organic acids and lower the pH of barley. Ferments (FS1, FS2 and FS3) appear to behave similarly after 48 h incubation. However, FS4 showed better acidifying power than the other ferments. This can be explained by the fact that there was a positive interaction between the three strains that stimulated this power and made the ferment FS4 the most active in terms of acidification. According to a mechanistic approach, this type of interaction is classified as an indirect positive interaction due to stimulation by a bacterial species that releases or produces specific metabolites in the medium, such as lactic acid, availability of nitrogen compounds, etc. In addition, LAB, particularly homolactic ones, have the capacity to produce a significant amount of lactic acid during their growth [17]. What's more, the four combinations were also tested for antagonistic activity, in vitro, against the same spoilage strains tested in pure culture.

From the results presented in (Table 3) and (Fig. 1), we can see that the inhibition of the ferment FS4 is the most remarkable compared to the other mixture, against all five spoilage strains tested. Its inhibitory effect reported to be most active after the elimination of the effect of hydrogen peroxide and the effect of organic acids from the cell-free supernatant. The strongest inhibition is reported against *E. coli*. While the least was observed against *Proteus vulgaris*.

Statistical analysis of antibacterial activity shows that there is a positive correlation between the number of strains and the inhibitory effect indicated at $P < 0.05$; the zone of inhibition widens as the number of strains increases. This inhibition is probably due to the production of antibacterial compounds of the bacteriocin type. In fact, previous studies have shown that all three strains making up the ferment FS4 produce bacteriocins. Nevertheless, the production of this compound depends on the bacterial species. Indeed, *L. plantarum* produces plantaricin [18] *Pediococcusspp* produces pediocin PA-1 [19] while *L. lactis* produces lactostrepcin, nisin, lacticin, dricin, and lactococcin [20]. Similarly, in a recent study of a total of 55 LAB isolated from fermented foods, results showed that (94%) of homofermentative LAB isolates exhibited broad-spectrum antimicrobial activity against food-borne pathogens, some of them producing bacteriocin [21]. It has also been shown that the growth of *E. coli* and *Salmonella enterica* is inhibited by the production of bacteriocins by a mixed culture of LAB [22]. Fijan, et al. reported that the agar antagonist activity of a probiotic containing multi-layers of LAB was more effective than a probiotic containing mono-layers of LAB against *E. coli* [23].

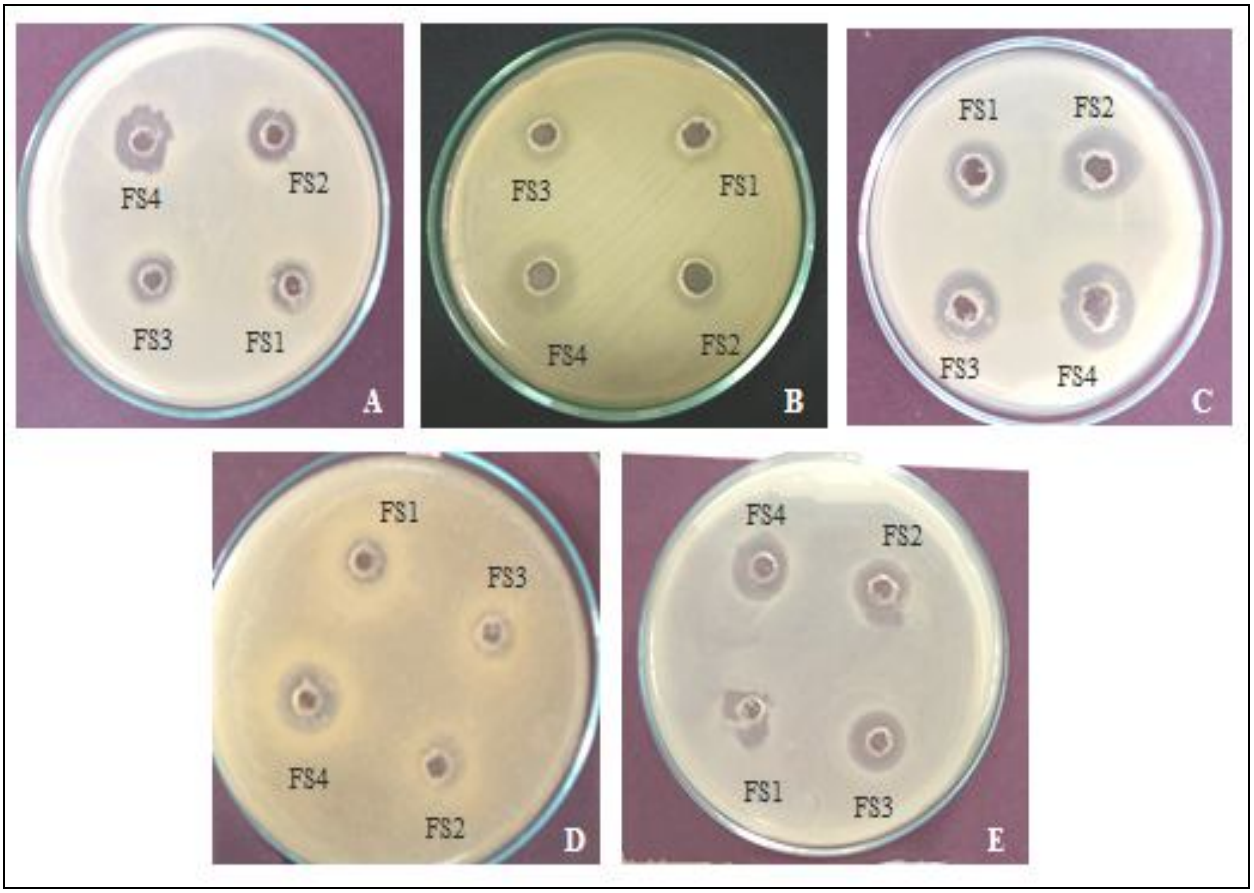

Figure 1. **Inhibition by four lactic ferments against five spoilage strains: (A)** ***Salmonella sp*****, (B)** ***Proteus vulgaris,*** **(C)** ***Escherichia Coli,*** **(D)** ***Staphylococcus aureus*****, and (E)** ***Klebsiella pneumonia.***

Table 3. **Antagonistic effect of four lactic ferments against five spoilage strains.**

	Inhibitory effect			
	FS1	***FS2***	***FS3***	***FS4***
Salmonella sp	+	++	+	+++
Proteus vulgaris	+	+	+	+++
Escherichia coli	++	+++	+++	+++
Staphylococcus aureus	+	+	+	+++
Klebsiella pneumoniae	+	++	+++	+++

(+) Presence of effect, (++) Medium effect, and (+++) Strong effect.

Fermentation trial

The controlled barley fermentation trial was initiated by inoculating 1 L of ferment into 4 kg of local barley. The mixture was agitated manually to avoid ferment deposits. The product was stored in a closed plastic barrel and placed in an insulated chamber and incubated at ambient temperature. The fermentation time was set as 15 days until the pH stabilized. The drying was carried out naturally, by distributing the barley uniformly over stainless-steel trays, then exposing it to the heat of the sun. The dry matter value of fermented barley is 76.25% after drying.

Monitoring of fermentation process control

Samples were taken regularly at T0, T5, T10 and T15. As a result, physicochemical and microbiological tests were carried out to monitor fermentation progress. We report a total absence of unpleasant odors in the product during fermentation due to the proper conduct of this process.

Physicochemical parameters
pH determination

The results of the pH control show that the pH changes versus time (Fig. 2). The initial pH of the fermentation trial was 5.89, and after 5 days, the pH value decreased to 4.22. The pH then began to stabilize towards the end of the fermentation cycle, with a final process pH of 4.12. These results are similar to those of another study carried out on barley inoculation with LAB, which showed that pH decreased after the sixth day of the experiment. The pH then stabilized from the fifteenth day until the end of the experiment, defined as 97 days [24].

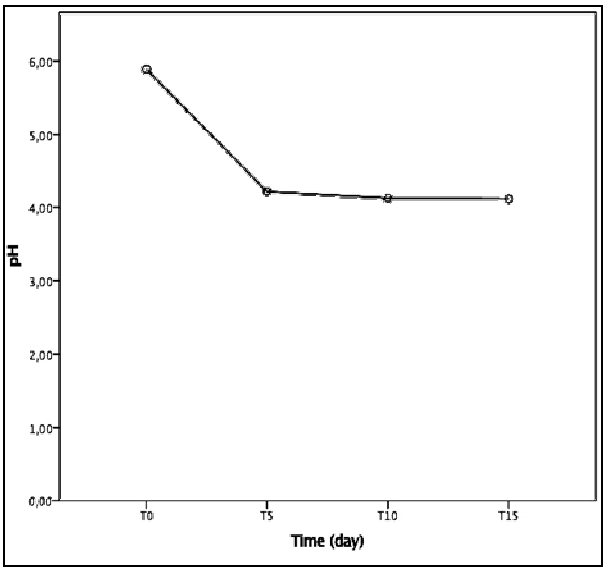

Figure 2. **Monitoring of pH evolution during the fermentation trial**

Temperature determination

The values recorded for this parameter fluctuated between 19.80 and 21.30°C during the barley fermentation trial. Although the temperature range recorded remains favorable to the growth and development of the three LAB making up the ferment FS4, it may also affect other factors such as enzyme activity and substrate availability.

Moreover, homolactic bacteria were characterized by increased production of lactic acid. The lower the temperature, the more lactic acid imparts strong acidity to the medium [25].

Moisture determination

The results of moisture monitoring of barley wetted with ferment FS4 showed that measured moisture values remained almost stable on all monitoring days. The average moisture value calculated over the trial period was 68.70±0.07%. After a natural drying process, the moisture content of the fermented barley is reduced to 23.75%. Aman et al. reported that hermetic storage of barley at high humidity results in significant changes in the solubility of proteins, starch and mixed-bond β-glucans. However, these changes did not show significant effects on nutritional properties, there being only small changes in gross chemical composition [26]. Others have shown that storing barley at high humidity (60% DM) reduces β-glucan levels in the grain and thus improves feed value [27].

Microbiological analysis

During the fermentation period, microbiological monitoring showed that germ populations were significantly affected, as shown in figures 3, 4 and 5 in CFU/g.

Total aerobic mesophilic flora count

The TAMF count indicates the hygienic quality of the product. The value recorded on the first day of the experiment was on average $281x10^5$ CFU/g. However, there was a reduction in TAMF germs towards the last day of fermentation. The estimated value on this day averaged $225x10^5$ CFU/g. The decrease in overall bacterial load growth of the sample is quite normal. The factors responsible are probably pH, and the inhibitory action exerted by substances from the secondary metabolism of LAB.

Coliform count

The total coliform count recorded on the first day of the experiment was on average $37x10^2$CFU/g. However, a total absence of TC was observed from the fifth day of fermentation (Fig. 4). This is probably due to the antibacterial effect induced by the LAB. For fecal coliforms, a total absence was observed in the product due to their absence in the source barley.

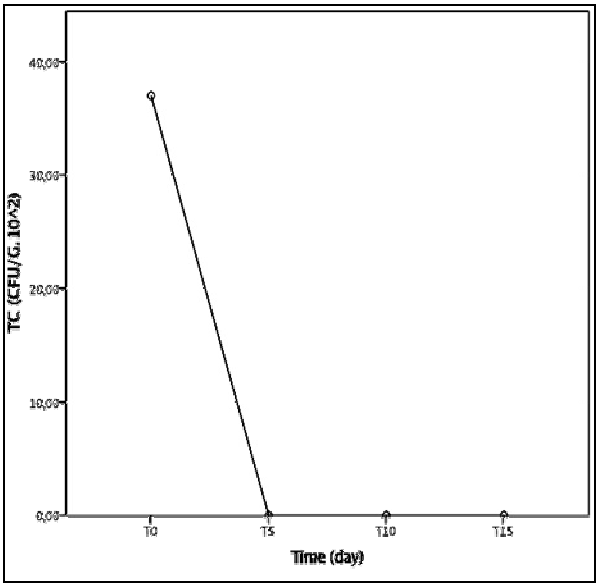

Figure 3. **Monitoring coliform count during the fermentation trial**

Fungal flora counts

At (T0), the fungal flora population averaged $610x10^2$ CFU/g.Afterwards, it decreased to $36x10^2$ CFU/g at the end of fermentation (Figure 5). This reduction is normal, since LAB have fungistatic properties that block the development and reproduction of yeasts, molds and fungi, and fungicidal properties that destroy them. This interpretation confirms the findings of Cizeikiene, et al, who reported that LAB are endowed with bacteriocin-like substances and produce organic acids that have characteristic fungistatic and fungicidal properties and inhibit fungi and yeasts, such as *Aspergillus versicolor, Debaryomyces hansenii, Penicillium expansum, Fusarium culmorum, A. fumigatus, Candida parapsilosis, A.niger* and *P.chrysogenum* [28].

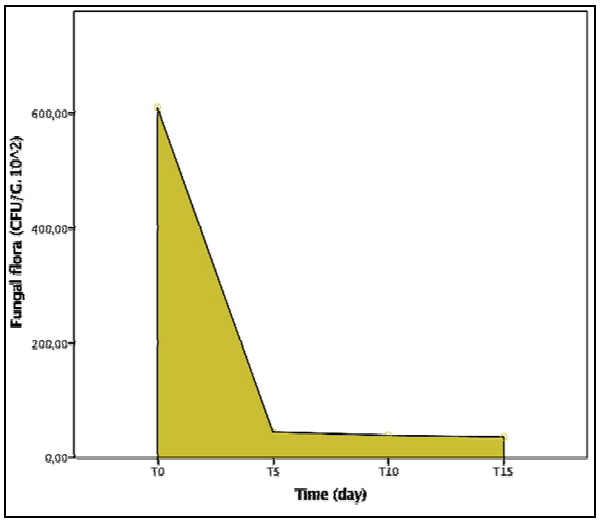

Figure 4. **Monitoringfungal flora count during the fermentation trial**

Lactic acid bacteria count

On the first day of fermentation, the results of the LAB count showed an average population of $1.19x10^4$ CFU/g. The microbial abundance increased thereafter, reaching a population of $1020x10^4$ CFU/g on the fifth day of fermentation. The growth of LAB then stabilizes towards the end of the fermentation cycle, with an abundance of $870x10^4$ CFU/g (Fig. 5). This abundance shows compatibility between the three strains explained by a synchronization of the fermentative metabolism of all the strains making up the fermentFS4.A phenomenological approach classifies this type of interaction as mutualism. In effect, each bacterium is stimulated by the presence of the other. This is referred to as a reciprocal, simultaneous or successive benefit relationship. This type of interaction can be observed when the growth of a first species brings modifications to the environment (pH change, elimination of inhibiting factors…), making it favorable to the growth of a second species [29].

The results of this work revealed that inoculation of the barley with the ferment FS4 favored a decrease in pH, with a modification in the overall microbial load of the product. Controlled homolactic fermentation lowered the pH from 5.89 (T0) to 4.12 after 15 days. This parameter is an indicator of the success of the process. The total absence of TC and the reduction in fungal flora is a sign of the inhibitory effect exerted by a mixed culture of the three strains; thanks to a mechanism of production of antibacterial metabolites, such as hydrogen peroxide, organic acids and bacteriocins. Statistical analysis showed that the mean difference was significant at the ($P \leq 0.05$) thresholds between LAB and TC, as well as between LAB and fungal flora. As a result, there was harmony between *L. plantarum, Pediococcus spp* and *L. lactis*.

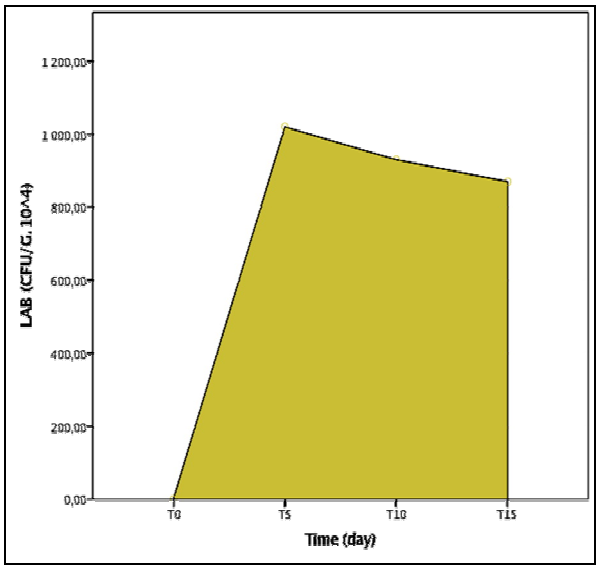

Figure 5. **Monitoring lactic acid bacteriacount during the fermentation trial**

Local barley contains a major carbohydrate reserve of starch averaging 53.5% dry matter; this content is generally inversely proportional to that of dietary fiber. Starch is considered a polysaccharide, and it is composed of glucose molecules linked with α-(1→4) and α-(1→6) glycosidic bonds that are easily broken down in the digestive tract of birds and mammals [30]. These bonds are resistant to animals' endogenous digestive enzymes [31] although they can be digested by microbe-derived enzymes [32]. While

dietary fibers contain sugars other than glucose or linkages other than α-(1→4) and α-(1→6), or both, an example of this carbohydrate is cellulose which forms part of the cell wall of plants, and it also contains non-starch polysaccharides, mainly β-glucans, which are closely associated with other polysaccharides or non-carbohydrate matter, such as proteins [32]. They thus form a mixed (1→3), (1→4)-β-glucan bond, and these associations will negatively influence the behavior of non-starchy polysaccharides when consumed by animals [30] Several studies have shown that the presence of barley β-glucans in animal feed is the main anti-nutritional factor, complicating digestibility and leading to a drop in zoo-technical performance [33, 34].

However, the elimination of the harmful effect of β-glucans from barley by LAB has been reported in various previous studies. Some have reported that lactobacilli have the capacity to reduce β-glucans [35]. Others have reported that barley ensiling (preservation by lactic acid fermentation of wet barley) reduces the harmful effects of β-glucans and increases nutrient digestibility [36]. The solubility of β-glucans can decrease during lactic acid fermentation, Skrede et al. reported that β-glucan degradation occurs in the early stages of fermentation controlled by LAB, and they also mentioned that this mechanism is probably due to the β-glucanase activity of the lactic strain used [33] Either way, controlled fermentation by LAB improves efficiency and can guarantee consistent hygienic and nutritional quality of a processed product. Silva et al, reported that the decomposition of barley fibrous components is caused by the addition of β-glucanase [37]. The optimum pH for the β-glucanase enzyme activity ranges between 5.5 and 7.5, while it decreases at a pH above 8.0 [38]. Habte et al. reported that the digestion of cereal grains with β-glucanase leads to maximum release of higher-quality protein and energy [39]. In fact, the β-glucanase enzyme is present in the cell walls of microorganisms, and it causes the hydrolysis of barley β-glucans; in particular mixed-linkage β-glucan bonds (1-3, 1-4) [40]. Apparently, the β-glucanase enzyme activity of LAB is probably responsible for eliminating the deleterious effects of β-glucans.

Conclusion

In light of these results, the mixed culture (FS4) of the strains *L. plantarum, Pediococcus spp* and *L. lactis* could be an effective and natural way to improve the hygienic and nutritional quality of barley. The fermented barley could be used as a valuable feed ingredient for animals, especially poultry and ruminants, as it would enhance their health and productivity. Future work could explore the effects of different fermentation conditions, such as temperature, time and moisture, on the quality and safety of barley fermented with the mixed culture (FS4). Moreover, animal trials could be conducted to evaluate the performance and health benefits of feeding fermented barley to different species.

Conflit of Interest

No conflit of interest.

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