The fermentability of agricultural raw materials by probiotic bacterial strains

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Abstract. The objective of this study was to evaluate the fermentability of various agricultural raw materials using a novel Liquid State Fermentation (LSF) technique. The formulations were based on protein-rich plant ingredients, such as sunflower, wheat, and rapeseed, addressing the persistent issue of byproducts in the food industry by seeking alternative utilization methods. While the LSF method has been used in pork production, it remains a new technology in the poultry sector. Distiller's Dried Grains with Solubles (DDGS) and Corn-Gluten Feed (CGF) were chosen based on previous experiments. These mixtures were enhanced by inoculation with various bacterial strains to produce fermented feeds with probiotic properties. The bacteria played a crucial role in the entire fermentation process. The starters included a commercial culture and fresh sweet whey of a semi-hard cheese. Additionally, selected bacterial strains were used based on previous research and literature data. Solaris model bioreactor system were utilized to produce the fermented feeds. This approach aims to promote a healthier gastrointestinal system in farm animals, protecting them against pathogenic bacteria. The fermentation process was designed to generate beneficial molecules such as enzymes, organic acids, and bacteriocins, further supporting the health benefits of the final product. This is significant because such feed can reduce the need for antibiotics in farm animal breeding, aligning with the EU's stance on minimizing antibiotic usage. Throughout our research, we meticulously monitored the fermentation process, gathering data for a comprehensive comparison. Our analysis focused on changes in pH, the microbiological and hygienic properties of the feed, and the production of organic acids in the fermenting mixtures. The results consistently showed a decrease in pH values after 24 h of fermentation. DDGS with selected strains exhibited the highest LAB counts at 9.89 $\log_{10}$ CFU/cm³, whereas the combination of CGF and whey produced the highest lactic acid concentration at 28.86 mg/ml. These promising results warrant further investigation through animal trials.

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1 Introduction

There is a continuous challenge in utilizing food-processing byproducts, which are protein-rich residues generated during the production of ethanol, starch, or cheese, such as Distillers Dried Grains with Solubles (DDGS), Corn Gluten Feed (CGF), and whey. These byproducts, an unavoidable result of certain food production processes, pose significant issues in terms of storage, logistics, and economics. A promising solution, as suggested by *Couto et al.* [1], is to repurpose these byproducts for feed production. This approach provides a pathway to creating high-quality, nutritionally rich feeds by incorporation certain protein crops. Liquid State Fermentation (LSF) represents a groundbreaking method in animal nutrition, offering the potential to dramatically enhance pig and poultry diets worldwide, thus benefiting producers and research institutions alike [2, 3]. This technique enables the use of less valuable feed materials, such as those with higher fiber content, to produce superior-quality end products by employing bacteria with probiotic properties.

The fermentation process enriches the feed's nutrient profile, diminishes antinutrients, and improves digestibility through microbial activity [4]. It leads to the production of organic acids, like lactic and acetic acids, causing a reduction in pH and enabling the proliferating bacteria to inhibit pathogens, positively affecting the animals' gut microbiome [5, 6]. The production of organic acids not only inhibits harmful bacteria but also decrease the pH, thereby preserving the feed quality for extended periods. This extended shelf life allows for greater storage capacity, potentially saving money for large companies.

The feeds developed through our research are expected to contain additional beneficial components such as enzymes, and bacteriocins that, both individually and synergistically, enhance farm animals' production performance [7, 8]. Fermented feeds play a crucial role in safeguarding the gastrointestinal health of animals, potentially reducing the necessity for antibiotics in compliance with EU regulations [9]. This approach aims to minimize the transmission of antibiotic resistance genes to humans, thereby reducing population exposure to such resistance.

2 Material and methods

2.1 Preparation of inocula and raw materials

The preparation of test samples and inocula took place at the Department of Food Science, Széchenyi István University, in Mosonmagyaróvár. The pre-cultivated Dr. Ferm culture, comprising a mix of *Lactiplantibacillus plantarum* and *Enterococcus faecium* strains, underwent cell counting with a DensiChek instrument, previously validated for these species. The final inoculum cell concentration was adjusted to approximately 10^5 CFU/cm^3 through a 200–250-fold dilution. A 50 cm^3 suspension was then prepared and introduced to the substrate at the start of the fermentation process, where the initial pH measurement was also performed.

The inoculation utilized *L. plantarum* DSMZ 16627 and *Pediococcus acidilactici* DSM 20284, employing 24–48 h pure cultures of the chosen strains. Whey, a byproduct from semi-hard Óvári-type cheese production, was sourced from the technological laboratory at the Department of Food Science, Széchenyi István University. Concurrently, the preparation methods for the raw materials were optimized in terms of consistency, pre-treatments, etc.

Following these adjustments, the pre-mixed dry ingredients were moistened with a diluted inoculum and heated at 55–60°C for 20 min. The resulting mixture, with a 22% dry matter content, was then inoculated and transferred into the fermentation system's reactors. Six different combinations were tested as shown in Table 1: basic sunflower, wheat, and rapeseed

supplemented with either DDGS or CGF, and three distinct inocula (Dr. Ferm bacterial culture, whey, and a mix of *L. plantarum* and *P. acidilactici* strains chosen by us). Each experiment was conducted in triplicate and the entire experimental program was repeated three times.

Table 1. Combinations of fermented feeds.

Ingredient	DDGS + Dr. Ferm	DDGS + selected strains	DDGS + whey	CGF + Dr. Ferm	CGF + selected strains	CGF + whey
Base (sunflower, wheat, rapeseed)						
Base	+	+	+	+	+	+
Byproduct						
DDGS	+	+	+	–	–	–
CGF	–	–	–	+	+	+
Inoculum						
Dr. Ferm	+	–	–	+	–	–
Selected strains	–	+	–	–	+	–
Cheese whey	–	–	+	–	–	+
Water						
Water	+	+	–	+	+	–

Notes: DDGS: Distillers Dried Grains with Solubles, CGF: Corn Gluten Feed.

2.2 Fermentation, sampling, and microbiological analysis

In this study, fermentation processes were conducted using Solaris model bioreactors (Solaris Biotech, Porto Mantovano, Italy), specifically employing a Jupiter 6.5-liter three-coupled reactor system alongside the Leonardo control software. Throughout the 24-h batch fermentation, stirring speeds were maintained between 500–800 rpm, and the temperature was held at 37 ± 1°C. These conditions, including pH, were monitored at 20-min intervals.

Following the fermentation process, samples from six distinct formulations were subjected to microbiological examination, followed by an analysis of their organic acid content, adhering to the protocols outlined in **Table 2**. To ensure precision, each sample was assessed in triplicate. The method involved transferring 1–1 cm^3 of stock suspension into Petri dishes with a sterile pipette, followed by subsequent decimal dilutions using fresh sterile pipettes for each dilution. For the specific assessment of viable *Enterococcus faecium* cell counts, 0.1 ml of the dilution was placed onto CATC agar plates according to standard procedures. The dilutions were then evenly spread over the agar surface using a sterile spreading tool, employing a circular motion to ensure uniform distribution.

Table 2. Standards for microbiological analyses.

Microorganism	Standard method
Enterococcus faecium	BVL L 06.00-32
Escherichia coli	ISO 16649-2:2001
Enterobacteriaceae	MSZ ISO 21528-2:2017
Coliforms	BS ISO 4832:2006
Clostridium perfringens	MSZ EN ISO 7937:2005
Lactobacillus / Mesophilic lactic acid bacteria	MSZ ISO 15214:2005
Salmonella spp. presence/absence	MSZ EN ISO 6579-1:2017

To estimate average cell counts, the following formula was applied (1):

$$\bar{C} = \frac{\Sigma c}{(n_1 + 0{,}1 \times n_2) \times V \times d} \quad (1)$$

where: Σc represents the total colonies counted across all selected plates, V is the volume plated (in ml), n_1 and n_2 denote the number of plates for the initial and subsequent evaluable dilutions, respectively, and d signifies the dilution factor.

Following cell count determination, a weighted arithmetic mean was calculated, along with the standard deviation to assess variability. Given that the data followed a normal distribution, continuous variables were analyzed using an unpaired t-test. An F-test was initially performed to compare variances across groups. Depending on the equality of variances, a homoscedastic (equal variance) or heteroscedastic (unequal variance) t-test was applied. A P value of less than 0.05 was deemed statistically significant.

2.3 Sample preparation for HPLC-UV analysis

For the qualitative and quantitative assessment of organic acids in fermentates, we employed an HPLC-UV system (Jasco, LC 900, Japan). The sample preparation protocol was designed following the Bio-Rad column recommendations for optimal fermentation monitoring. The centrifugation process was executed in two stages:

(1) Initial centrifugation: a volume of 15 ml from each fermentate was transferred into centrifuge tubes and subjected to centrifugation at 6,000 rpm for 20 min using a Hermle Z206 A centrifuge (Germany).
(2) Secondary centrifugation: from the above supernatant, 1.5 ml was pipetted into Eppendorf tubes and further centrifuged at 14,500 rpm for 10 min using a Biosan Microspin 12 centrifuge (Latvia).

The clarified supernatant obtained was then passed through a 0.22 µm hydrophilic syringe filter directly into 1.5 ml HPLC vials equipped with septum screw caps for analysis.

For the separation of organic acids, we utilized a Bio-Rad Aminex HPX-87H column maintained at 50°C. The mobile phase comprised a 0.005 M H_2SO_4 solution, delivered at a flow rate of 0.8 ml/min. Detection of organic acids was conducted using a UV detector set to a wavelength of 220 nm, with an injection volume of 20 µl for each sample, following the method described by *Wang et al.* [10].

The analysis targeted several organic acids, including lactic acid, acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid, and isovaleric acid present in the fermentates. Calibration for these acids was established using individual standard solutions at 10 mg/ml, followed by preparation of a series of dilutions to span a concentration range of 0.05–10 mg/ml. Calibration solutions were prepared as shown in **Table 3**, with all solutions being adjusted with a 0.005 M H_2SO_4 solution to ensure accurate signal detection.

This meticulous approach to sample preparation and calibration ensured precise quantification and analysis of the organic acid content in fermentates.

Table 3. Preparation of calibration solutions for organic acids

Concentration (mg/ml)	Source solution	Volume pipetted (ml)	Final volume (ml)
1	Stock solution	1	10
0.5	1 mg/ml	2.5	5
0.2	1 mg/ml	1	5
0.1	1 mg/ml	0.5	5
0.05	1 mg/ml	0.25	5

3 Results and discussion

3.1 Changes in pH values during the fermentation process

The utilization of the Leonardo software system within the Solaris bioreactor facilitated precise monitoring of pH values throughout the fermentation process. This enabled a thorough comparison of data collected during the experiment. It was observed that all final products exhibited lower pH levels compared to their initial mixtures. This decrease in pH could be attributed to the production of lactic acid by lactic acid bacteria (LAB). The results of these observations are illustrated in **Figure 1**.

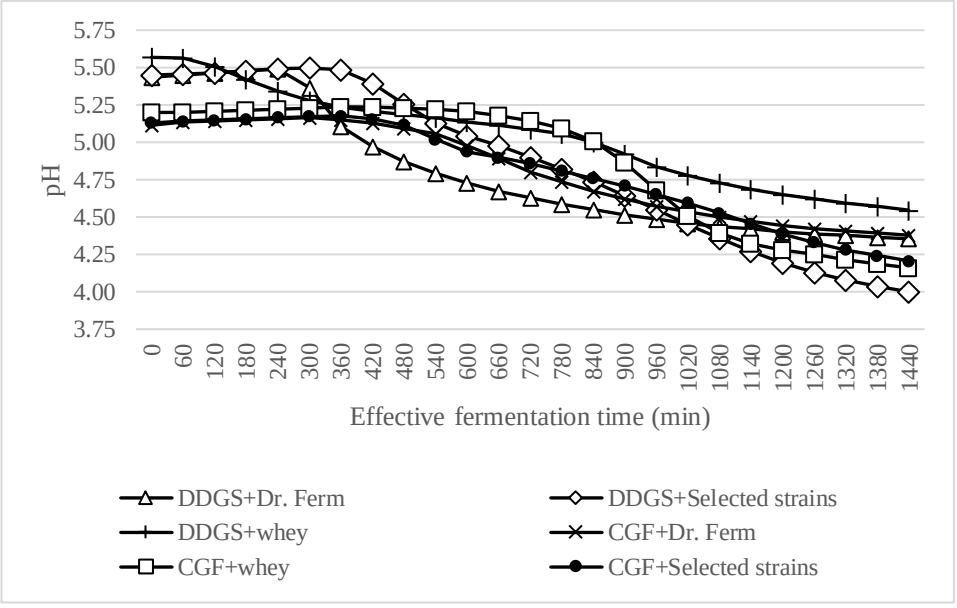

Fig. 1. Changes in pH values throughout the fermentation process.

Throughout the fermentation, all experimental conditions were kept constant, including temperature and inoculated cell numbers. Therefore, the variations in pH values were due to the differing buffer capacities of the base materials and their impact on the microbial strains. No notable differences in pH were observed between the DDGS + Dr. Ferm and CGF + Dr. Ferm combinations, as well as between the CGF + whey and CGF + selected strains mixtures. Conversely, in all other comparisons, the differences in final pH values were significant ($P<0.05$). The combination of DDGS + selected strains was found to have the lowest average pH value.

3.2 Microbiological and hygienic properties of fermented feed

The assessment of the microbiological and hygienic quality of raw materials for fermentation and the resulting fermented products identified several microbial groups outlined in the Feed Regulation [Order 65/2012 (VII. 4.) by the Ministry of Rural Development]. These groups included *Enterococcus faecium, Escherichia coli, Enterobacteriaceae, coliforms, Clostridium perfringens, Lactobacillus* / mesophilic LAB, and *Salmonella spp.* Our findings revealed the absence of *Salmonella spp.* in any final fermentation products. Additionally,

Escherichia coli, Clostridium perfringens, and *Enterobacteriaceae* species levels were below detectable limits. *Coliform* species were only identified in the DDGS + whey and CGF + Dr. Ferm combinations, with concentrations of 104 CFU/ml and 102 CFU/ml, respectively, across all parallels. The DDGS + selected strains combination showed the highest count of LAB at 9.89 log10 CFU/ml, as indicated in **Table 4**. According to Wendel [11], probiotic benefits are noticeable at concentrations exceeding tens of millions of CFU per gram, suggesting the selected combination warrants further in vivo animal studies. Moreover, the DDGS + Dr. Ferm blend showed superior levels of *Enterococcus faecium*, known for its excellent organic acid production capabilities [12].

Table 4. Post-fermentation viable cell counts*.

Bacterial species	Fermentation recipe					
	DDGS + Dr. Ferm	DDGS + selected strains	DDGS + whey	CGF + Dr. Ferm	CGF + selected strains	CGF + whey
Lactiplanti-bacillus plantarum	9.24 ± 0.04^{b}	9.89 ± 0.05^{a}	9.32 ± 0.02^{b}	9.08 ± 0.07^{c}	9.70 ± 0.21^{a}	8.33 ± 0.54^{d}
Enterococcus faecium	9.26 ± 0.02^{a}	6.07 ± 0.63^{b}	6.56 ± 1.39^{b}	7.92 ± 1.14^{b}	2.61 ± 0.64^{d}	3.32 ± 0.62^{c}

Notes: $^{a–d}$ Means within a row without a common superscript differ ($P<0.05$). * Values are $\log_{10}$ CFU/ml means ± SD, based on nine observations (three samples, three replicates). DDGS: Distillers Dried Grains with Solubles, CGF: Corn Gluten Feed.

3.3 Presence of organic acids

The analysis of organic acids was conducted using the HPLC-UV method, focusing on the chromatographic peaks at a wavelength of 200 nm. This specific wavelength was chosen due to its correspondence with the maximum absorption for the organic acids of interest. The presence of organic acids was confirmed in all tested samples, indicating their significant absorption at this wavelength. The findings are summarized in **Table 5**.

Table 5. Organic acid concentrations* in samples analyzed by HPLC–UV.

Sample	Organic acid concentration (mg/ml)			
	Lactic acid	Acetic acid	Propionic acid	Isobutyric acid
DDGS + Dr. Ferm	13.08 ± 0.79^{d}	3.75 ± 0.76^{a}	2.92 ± 0.18^{b}	Not detected
DDGS + strains	20.23 ± 2.56^{b}	0.89 ± 0.05^{a}	2.01 ± 0.25^{c}	0.27 ± 0.07^{b}
DDGS + whey	15.85 ± 2.39^{c}	2.60 ± 1.15^{a}	2.02 ± 0.41^{c}	0.35 ± 0.11^{b}
CGF + Dr. Ferm	15.43 ± 1.01^{c}	1.16 ± 0.33^{a}	4.81 ± 0.21^{a}	0.46 ± 0.11^{b}
CGF + strains	20.74 ± 2.57^{b}	1.20 ± 0.48^{a}	4.86 ± 0.77^{a}	0.47 ± 0.12^{b}
CGF + whey	28.86 ± 1.97^{a}	2.61 ± 0.50^{a}	4.67 ± 0.22^{a}	1.00 ± 0.04^{a}

Notes: $^{a–d}$ Means within a column without a common superscript differ ($P<0.05$). * Values are means ± SD, based on nine observations (three samples, three replicates). DDGS: Distillers Dried Grains with Solubles, CGF: Corn Gluten Feed.

The goodness of fit for the calibration equations is indicated by the R^2 value, which shows how closely each measurement point fits the linear correlation coefficient (R). For a perfect line fit, R is 1. Our calibration values are presented in Table 6.

Table 6. Linearity of the curves, limit of detection, and limit of quantitation.

Component	R^2	LOD (mg/ml)	LOQ (mg/ml)
Lactic acid	0.999	0.016	0.053
Acetic acid	1	0.011	0.039
Propionic acid	1	0.009	0.041
Isobutyric acid	1	0.019	0.052

The HPLC-UV analysis confirmed the presence of lactic, acetic, propionic, and isobutyric acids across the samples (Figure 2). Notably, the CGF + whey combination exhibited the highest concentrations of these acids. Isobutyric acid was not detected only in the DDGS + Dr. Ferm sample, highlighting a unique distinction in its organic acid profile compared to the other combinations.

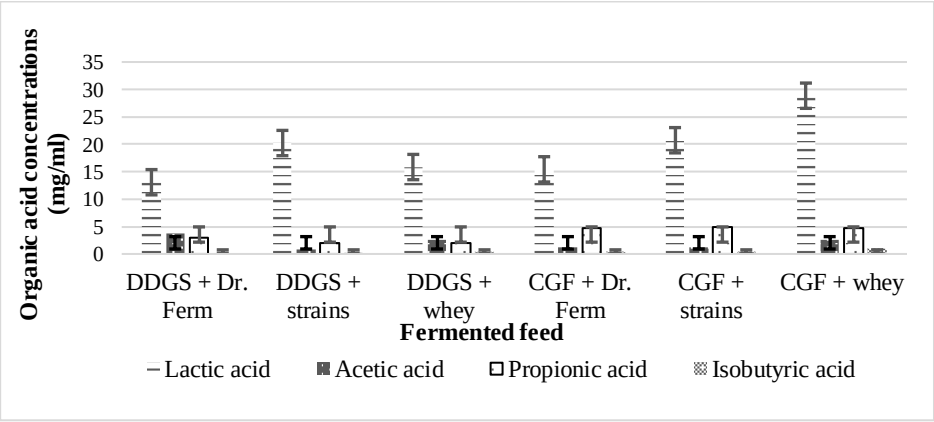

Fig. 2. Organic acid concentrations of fermented feeds.

4 Conclusions and suggestions

In all mixtures tested, there was a consistent decrease in the average pH level 24 h after initiation. This trend was linked to the presence of at least 10 mg/ml of lactic acid in each sample, which contributed to the reduction in pH levels. Notably, the combination of DDGS with selected bacterial strains demonstrated the most substantial drop in mean pH values. The microbiological analysis revealed that this specific combination harbored the highest concentration of LAB, positioning it as an excellent candidate for future animal studies. However, the DDGS + Dr. Ferm mixture showed potential for later phases of testing, due to its high levels of *Enterococcus faecium*, which also played a role in acid production. Among the tested combinations, CGF + whey stood out by producing the highest amounts of lactic acid (28.86 mg/ml) and isobutyric acid (1.00 mg/ml). The highest concentration of propionic acid was found in the CGF + selected strains ferment (4.86 mg/ml), whereas the DDGS + Dr. Ferm feed led to increased acetic acid production (3.75 mg/ml). These organic acids are crucial for reducing pH levels, which benefits feed storability by potentially extending shelf life. This aspect is critical for maintaining the feed's quality over time. Our findings offer promising prospects for animal testing. The elevated numbers of LAB, including *Enterococcus faecium*, along with the significant production of organic acids, could provide effective protection for the gastrointestinal system of farm animals, enhancing their overall health and productivity. Further trials are required to determine the influence of our probiotic

fermented feeds on broiler chickens' daily weight gain, gut microbial status, and meat quality to gain a comprehensive understanding of their overall efficiency.

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