Research on the Bacterial Fermentation of Peanut Meal and Their Effects on the Production Performance of Broilers

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Abstract

Peanut meal (PNM), a byproduct of the peanut oil extraction process, is a valuable source of protein and energy in animal feed. Nevertheless, its utility is hindered by an uneven amino acid profile, particularly concerning essential amino acids such as Lys, Met, and Thr. PNM also contains anti-nutritional factors (ANFs) that negatively impact its nutritional value and digestibility. For instance, phytic acid in PNM forms complexes with minerals, reducing their bioavailability to animals. The high fiber content in PNM can also lead to digestive issues and reduced nutrient absorption. Furthermore, PNM is susceptible to contamination by potent toxins known as aflatoxins, produced by *Aspergillus fungi*, posing a risk to animal health. In addressing these challenges, fermentation techniques exhibit promise, having demonstrated their capacity to enhance feed ingredient nutritional value by degrading ANFs, optimizing amino acid profiles, and augmenting nutrient accessibility. Additionally, fermentation can yield beneficial metabolites and enzymes that bolster animal health and improve performance.

To initiate fermentation, the selection of appropriate microorganisms is crucial. A strain of *Bacillus velezensis* LB-Y-1 was screened based on the nutritional characteristics of PNM. This strain, identified through a targeted screening regimen, showcased remarkable potential for the production of multiple enzymes, including protease, cellulase, and phytase. This capability enabled it to break down large protein molecules, convert amino acids, and reduce cellulose and phytic acid content in PNM. Additionally, incorporating LB-Y-1 into the diet positively influenced the growth performance and tibia mineralization of chicken broilers. Analysis of the intestinal microbiota revealed an abundance of beneficial genera such as *Parasutterella* and *Rikenellaceae*, while the opportunistic pathogen *Escherichia-Shigella* was significantly reduced in the LB-Y-1 supplemented group. These findings collectively suggest that LB-Y-1 holds potential as a viable strain for further applications as a starter culture for fermentation processes.

Furthermore, we also screened a potential probiotic strain LC-9-1, which was screened from the intestines of healthy animals, was identified as *Pediococcus acidilactici*. This strain exhibited exceptional properties, such as efficient acid production, antibacterial activity, and antioxidant capability. When applied in the fermentation of PNM, it effectively reduced the pH of the fermentation product, resisted contamination from pathogenic microorganisms, and enhanced the product's antioxidant capacity. Moreover, experimental studies conducted on broilers revealed that dietary supplementation with LC-9-1 was safe. Additionally, it led to a reduction in abdominal fat deposition and improvements in antioxidant capacity and intestinal immunity in broilers.

The solid-state fermentation was conducted by utilizing the aforementioned bacteria and employing conventional techniques. Significant changes in the

nutritional composition of PNM were observed after fermentation. The levels of crude protein, TCA-soluble protein, and L-lactic acid were significantly increased, while the concentrations of crude fiber, phytic acid, and aflatoxin B_1 were notably decreased. In addition, solid-state fermentation resulted in an increase in the free amino acid content and improved the balance of hydrolyzed amino acids in PNM. We conducted an assessment of the nutritional value of FPNM in broilers. The results showed that feeding on FPNM resulted in higher apparent ileal digestibility (AID) and standardized ileal digestibility (SID) values for essential amino acids, including Met, Lys, Leu and Phe. Additionally, the AID and SID values for non-essential amino acids in FPNM were higher compared to PNM, except for Pro. However, fermentation did not have a significant effect on the apparent metabolizable energy (AME) value.

In the final study, a feeding trial was conducted, where different gradients of PNM and FPNM (5%, 10%, and 15%) were added to broiler diets. The results revealed that compared to the corn-soybean meal diets, supplementation of 10% PNM led to decreased levels of Lys, Met, and Thr in the breast muscle. However, the supplementation of 10% FPNM mitigated these changes. Furthermore, the broilers in the 10% FPNM group exhibited notable improvements in meat quality parameters such as meat color (redness, a*), $pH_{24\ hour}$ values, and oxidative stability. Additionally, the 10% FPNM group showed enhanced antioxidant capacity, as indicated by higher levels of total antioxidant capacity (T-AOC) and superoxide dismutase (SOD), along with lower levels of malondialdehyde (MDA) compared to the 10% PNM group. In conclusion, FPNM positively influenced broiler growth performance, meat quality, and oxidative stability. Taking all performance indicators into consideration, the optimal outcomes were obtained when the addition amount of FPNM was 10%.

Keywords: peanut meal, fermentation, broilers, *B. velezensis*, *P. acidilactici*, ileal digestibility of amino acids, apparent metabolizable energy, meat quality

Résumé

La farine d'arachide (PNM), sous-produit du processus d'extraction de l'huile d'arachide, constitue une précieuse source de protéines et d'énergie dans l'alimentation animale. Cependant, son utilité est entravée par un profil en acides aminés inégal, en particulier en ce qui concerne les acides aminés essentiels tels que la Lys, la Met et la Thr. Le PNM contient également des facteurs antinutritionnels (ANFs) qui ont un impact négatif sur sa valeur nutritionnelle et sa digestibilité. Par exemple, l'acide phytique contenu dans le PNM forme des complexes avec les minéraux, réduisant leur biodisponibilité pour les animaux. La teneur élevée en fibres du PNM peut également entraîner des problèmes digestifs et une absorption réduite des nutriments. De plus, le PNM est susceptible d'être contaminé par de puissantes toxines appelées aflatoxines, produites par les champignons *Aspergillus*, ce qui constitue un risque pour la santé animale. Pour faire face à ces défis, les techniques de fermentation montrent des promesses, ayant démontré leur capacité à améliorer la valeur nutritionnelle des ingrédients pour l'alimentation animale en dégradant les ANFs, en optimisant les profils en acides aminés et en augmentant l'accessibilité des nutriments. De plus, la fermentation peut produire des métabolites et des enzymes bénéfiques qui renforcent la santé animale et améliorent les performances.

Pour initier la fermentation, la sélection de micro-organismes appropriés est cruciale. Une souche de *Bacillus velezensis* LB-Y-1 a été sélectionnée en fonction des caractéristiques nutritionnelles du PNM. Cette souche, identifiée grâce à un protocole de sélection ciblé, a démontré un potentiel remarquable pour la production de multiples enzymes, notamment la protéase, la cellulase et la phytase. Cette capacité lui a permis de décomposer de grandes molécules de protéines, de convertir les acides aminés et de réduire la cellulose ainsi que la teneur en acide phytique dans le PNM. De plus, l'incorporation de LB-Y-1 dans l'alimentation a eu un impact positif sur les performances de croissance et la minéralisation du tibia des poulets de chair. L'analyse de la microbiote intestinale a révélé une abondance de genres bénéfiques tels que *Parasutterella* et *Rikenellaceae*, tandis que le pathogène opportuniste *Escherichia-Shigella* était significativement réduit dans le groupe supplémenté en LB-Y-1. Ces découvertes suggèrent collectivement que LB-Y-1 présente un potentiel en tant que souche viable pour d'autres applications en tant que culture de départ pour les processus de fermentation.

De plus, nous avons également sélectionné une souche potentielle de probiotiques, LC-9-1, qui a été isolée des intestins d'animaux en bonne santé et identifiée comme *Pediococcus acidilactici*. Cette souche a présenté des propriétés exceptionnelles, telles qu'une production efficace d'acide, une activité antibactérienne et une capacité antioxydante. Lorsqu'elle a été utilisée dans la fermentation du PNM, elle a efficacement réduit le pH du produit de fermentation,

résisté à la contamination par des micro-organismes pathogènes et amélioré la capacité antioxydante du produit. De plus, des études expérimentales menées sur des poulets ont révélé que la supplémentation alimentaire en LC-9-1 était sûre. De plus, elle a entraîné une réduction du dépôt de graisse abdominale et des améliorations de la capacité antioxydante et de l'immunité intestinale chez les poulets.

La fermentation en état solide a été réalisée en utilisant les bactéries mentionnées précédemment et en employant des techniques conventionnelles. Des changements significatifs dans la composition nutritionnelle de la PNM ont été observés après la fermentation. Les niveaux de protéines brutes, de protéines solubles dans l'acide trichloroacétique (TCA-SP) et d'acide L-lactique ont augmenté de manière significative, tandis que les concentrations de fibres brutes, d'acide phytique et d'aflatoxine B1 ont notablement diminué. De plus, la fermentation en état solide a entraîné une augmentation de la teneur en acides aminés libres et amélioré l'équilibre des acides aminés hydrolysés dans la PNM. Nous avons effectué une évaluation de la valeur nutritionnelle du FPNM chez les poulets de chair. Les résultats ont montré que l'alimentation à base de FPNM entraînait une digestibilité iléale apparente (AID) plus élevée et des valeurs de digestibilité iléale standardisée (SID) plus élevées pour les acides aminés essentiels, tels que la Met, la Lys, la Leu et la Phe. De plus, les valeurs d'AID et de SID pour les acides aminés non essentiels dans le FPNM étaient plus élevées par rapport à la PNM, à l'exception de la Pro. Cependant, la fermentation n'a pas eu d'effet significatif sur la valeur d'énergie apparente métabolisable (AME).

Dans l'étude finale, un essai d'alimentation a été mené, où différentes concentrations de PNM et de FPNM (5%, 10% et 15%) ont été ajoutées aux régimes alimentaires des poulets de chair. Les résultats ont révélé que par rapport aux régimes à base de maïs et de tourteau de soja, la supplémentation en PNM à hauteur de 10% entraînait une diminution des niveaux de Lys, Met et Thr dans le muscle de la poitrine. Cependant, la supplémentation en FPNM à 10% atténuait ces changements. De plus, les poulets du groupe à 10% de FPNM présentaient des améliorations notables dans les paramètres de qualité de la viande tels que la couleur de la viande (rougeur, a*), les valeurs de pH à 24 heures et la stabilité oxydative. De plus, le groupe à 10% de FPNM présentait une capacité antioxydante renforcée, comme en témoignent des niveaux plus élevés de capacité antioxydante totale (T-AOC) et de superoxyde dismutase (SOD), ainsi que des niveaux plus faibles de malondialdéhyde (MDA) par rapport au groupe à 10% de PNM. En conclusion, le FPNM a eu un impact positif sur les performances de croissance des poulets de chair, la qualité de la viande et la stabilité oxydative. En prenant en compte tous les indicateurs de performance, les résultats optimaux ont été obtenus avec une supplémentation de 10% de FPNM.

Mots-clés: farine d'arachide, fermentation, poulets de chair, *B. velezensis*, *P. acidilactici*, digestibilité iléale des acides aminés, énergie métabolisable apparente, qualité de la viande.

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Chong Li

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PNM: Peanut meal
FPNM: Fermented peanut meal
BW: Body weight
ADFI: Average daily feed intake
ADG: Average daily gain
FCR & F/G: Ratio of feed intake / weight gain
FI: Feed intake
PUFA: Polyunsaturated fatty acids
TC: Total cholesterol
TG: Triglycerides
GSH: Glutathione
T-AOC: Total anti-oxidant capacity
SOD: Superoxide dismutase
GSH-Px: Glutathione peroxidase
MDA: Malonaldehyde
CAT: Catalase
***P. Mirabilis*:** *Proteus mirabilis*
***S. Aureus*:** *Staphylococcus aureus*
***E. Coli*:** *Escherichia coli*
V/C ratio: Villus length (μm)/Crypt depth (μm)
CON: Control group
BV: *Bacillus velezensis*
PA: *Pediococcus acidilactici*
LABs: Lactic acid bacteria
RBC: Red blood cell count
WBC: White blood cell count
LYM: Lymphocytes
HGB: Hemoglobin concentration
ALP: The alkaline phosphatase
AST: Aspartate aminotransferase
ALT: Alanine aminotransferase
HGB: Hemoglobin concentration
ALT: Alanine aminotransferase
AST: Aspartate aminotransferase
TP: Total protein
A/G ratio: Albumin / Blobulin
AID: The apparent ileal digestibility
SID: The standardized ileal digestibility
AME: The apparent metabolizable energy

ARGE: The apparent retention of gross energy
DM: Dry matter
CP: Crude protein
CF: Crude fiber
EE: Crude fat
AFB_1: Aflatoxin B_1
TCA-SP: Trichloroacetic-acid-soluble protein
Ala: Alanine
Arg: Arginine
Asp: Aspartic acid
Cys: Cysteine
Glu: Glutamic acid
Gly: Glycine
His: Histidine
Ile: Isoleucine
Leu: Leucine
Lys: Lysine
Met: Methionine
Phe: Phenylalanine
Pro: Proline
Ser: Serine
Thr: Threonine
Trp: Tryptophan
Tyr: Tyrosine
Val: Valine
NFD: Nitrogen-free diet
UAA: Umami amino acids
EAA: Essential amino acids
IMP: Inosinic acid, inosine 5'-monophosphate

Chapter I

General introduction

Chapter I. General introduction

Poultry production plays a vital role in meeting the global demand for meat products, especially poultry meat, which is widely consumed due to its high nutritional value, convenience, and affordability (Fan et al., 2018). The increasing population, urbanization, and changing dietary patterns have resulted in a surge in poultry consumption worldwide. As a result, the poultry industry has faced the challenge of meeting the growing demand while ensuring sustainable and efficient production practices (Fletcher et al., 2000; Wang et al., 2017b).

One crucial aspect of poultry nutrition is the formulation of balanced diets that meet the dietary requirements of birds for optimal growth, development, and health (Teng et al., 2021). Proteins are essential components of poultry diets as they provide the necessary amino acids for muscle development, organ function, and overall performance (Couri et al., 2000; Wang et al., 2019; Lu et al., 2023). However, the conventional protein sources used in poultry feed, such as soybean meal and fish meal, face limitations due to their competition with human food supply, environmental concerns, and fluctuating prices (Gilbert et al., 2017). Taking China as an example, in 2021, the feed grain consumption reached 395 million tons, accounting for 48% of the total grain consumption in the country, posing a significant threat of competition between human and animal food resources (Ministry of Agriculture, Beijing, China 2022). Moreover, traditional poultry feed formulations in China heavily rely on corn and soybean meal, with a particularly high demand for soybean meal. In terms of modern poultry production system, feed costs constitute approximately 75% of the total costs, with soybean meal accounting for around 25% of the feed costs (Mbukwane et al., 2022). In recent years, protein feed ingredients have experienced substantial price increases influenced by factors such as global economy, politics, and climate. Taking the selling price of soybean meal in China as an example, the average price in 2022 was $ 642.85 per ton (Source: https://finance.sina.com.cn/money/future/wemedia/2023-02-08/doc-imyeynxs6437643.shtml), representing an increase of approximately 25.0 % compared to $ 514.29 per ton in 2021. This change significantly impacts poultry farming costs. Additionally, China has limited land resources suitable for soybean cultivation, resulting in a high dependence on soybean imports. According to data released by the General Administration of Customs of China (2022), China imported 96.518 million tons of soybeans out of a total of 164.539 million tons of grain imports in 2021, while domestic soybean production was only 16.4 million tons. Based on the above, it is evident that exploring and utilizing domestic protein feed resources, improving the utilization of unconventional protein feed ingredients, is of significant importance. This approach can help reduce the proportion of soybean meal in feed formulations and lower the overall cost of poultry production.

Various countries have undertaken extensive research and development efforts to identify and evaluate alternative protein sources for poultry nutrition. China, as the world's largest producer and consumer of poultry meat, faces unique challenges and opportunities in this regard, driven by the increasing demand for meat products, has placed significant pressure on feed resources and protein availability. Therefore, it is imperative to explore and implement innovative strategies for sustainable poultry production in China, including the utilization of non-competitive protein sources. Researchers and industry professionals have been investigating alternative protein sources for poultry diets. The utilization of feed ingredients or by-products that do not directly compete with human food resources has garnered significant attention. Positive findings have been observed in the biological treatment of cottonseed meal (He et al., 2015), rapeseed meal (Wang et al., 2019; Wu et al., 2020), low-glucosinolate rapeseed meal (canola meal) (Ahmed et al., 2016), and palm kernel cake (Alshelmani et al., 2016), among others. By exploring and incorporating these alternative ingredients into poultry diets, the industry can achieve a more balanced and sustainable approach to feed formulation, contributing to the overall development of the poultry sector.

Peanut meal (PNM), as a by-product of oil extraction, has an annual production in China of approximately 4.5-5.9 million tons (Zhao et al., 2012). Its price is relatively lower and more stable compared to soybean meal, with recent average prices hovering around $ 401.57 per ton. Therefore, PNM holds great potential as a traditional protein substitute. However, the practical challenge lies in the nutritional and non-nutritional limitations associated with its application as a poultry feed ingredient (Gupta, 1987; Batal et al., 2005; Tola and Kebede, 2016). Hence, it becomes crucial to employ specific technological approaches to enhance its suitability for poultry feed utilization.

Another driving force behind our research is the prevalent concern over low-quality meat in poultry. Various factors contribute to this issue, including genetic factors, inadequate nutrition, improper management practices, and suboptimal feed formulations. These factors can result in undesirable meat quality characteristics such as pale color, soft texture, and reduced juiciness, which adversely affect consumer acceptance and market value (Petracci and Cavani, 2011). In our investigation of the nutritional composition of PNM, we have observed that while certain essential amino acids (such as Met, Lys, and Thr) may be limited in this ingredient, it contains higher levels of flavour amino acids (such as Arg, Glu, and Gly) (Council, 1994). Additionally, PNM harbor antioxidant peptides, flavonoids, and phenolic compounds that exhibit antioxidative properties, we believe that these components can positively impact the health and meat quality of poultry.

This study aims to advance our understanding of poultry nutrition and explore the utilization of alternative protein sources in broiler diets. Specifically, our research will focus on the solid-state fermentation of PNM using selected bacteria, followed by a comparative analysis of the changes that occur in PNM before and after fermentation. Furthermore, we will investigate the effects of supplementing different proportions of PNM and fermented PNM in broiler diets, assessing their

impact on growth performance, antioxidant capacity, meat quality, and oxidative stability. By evaluating these parameters, we can provide valuable insights into the potential benefits and feasibility of utilizing alternative protein sources in poultry diets, both globally and within the context of China.

Chapter II

Review of the literature

This chapter is based on the following publication:

Chong Li, Shuzhen Li, Yanbin Zhu, Si Chen, Xiaoying Wang, Xuejuan Deng, Guohua Liu, Yves Beckers and Huiyi Cai

Improving the Nutritional Value of Plant Protein Sources as Poultry Feed through Solid-State Fermentation with a Special Focus on Peanut Meal—Advances and Perspectives

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Chapter II. Improving the nutritional value of plant protein sources as poultry feed through solid-State fermentation with a special focus on peanut meal-a review

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Key words: peanut meal, solid-state fermentation, amino acid, anti-nutritional factor, plant protein source, poultry

1. Abstract

The poultry industry has been and is still suffering considerable challenges because of the increasing price of soybean meal. Therefore, it is imperative to find alternative, high-quality plant protein sources. Peanut meal (PNM), a by-product of peanut oil extraction, is abundant in crude protein (40.1-50.9%), making it a potential plant protein source. However, nutritional and non-nutritional limitations are detrimental to its application in poultry diets, such as an imbalance in amino acid composition, phytate and the risk of aflatoxins pollution. As a processing technique, solid-state fermentation has been used to reduce phytate and improve the nutrient availability of plant protein sources in the feed industry. It is a promising approach to improving the application of PNM in poultry diets. There are several advantages to the solid-state fermentation of PNM, such as low-cost equipment, high productivity, the stability of the product and the minimization of energy consumption. Currently, there is still a lack of synthesized information on the application of solid-state fermented PNM in poultry. This review summarized the limiting factors for PNM application in poultry feed and the improvement of solid-state fermentation on the nutritional value of plant protein sources so as to evaluate the feasibility of improving the nutritional value of PNM as poultry feed through solid-state fermentation. We hope to shed some light on the selection of protein resources in future research.

2. Introduction

As one of the world's five major oil seeds, peanuts are widely cultivated around the world. China is the largest peanut producing country with the production of 18.3 million tons in 2022, followed by India, Nigeria and the United States with 6.7, 4.5 and 2.5 million tons, respectively (United States Department of Agriculture (USDA)., 2022). However, there are great differences in the utilization of peanuts in different regions due to the differences in dietary habits. In the United States, nearly 60% of peanuts are used for direct consumption, and in Europe, the proportion is more than 90%. In the case of China, the proportion of peanuts used for food is less than 40%, and more than 50% (9 million tons) are used for the extraction of peanut oil because the Chinese prefer to stir-fry their food with it (Fletcher and Shi, 2016). The peanut oil is produced mainly by high temperature pressing and solvent extraction, which will produce 50–65% of residue (peanut meal, PNM), which appears as massive, powdery or flake-like (Figure 1) (Zhao et al., 2012). Thus, 4.5–5.9 million tons of PNM per year will be obtained in China, and it will bring huge economic benefits if this by-product is fully utilized. The nutritional value of PNM has been well clarified, with 40.1–50.9% crude protein, 0.7–6.0% fat and 5.8–12.6% fiber, as well as a rich array of vitamins, minerals and antioxidant components. Furthermore, it also contains some active components, such as resveratrol and peanut lectin (Sales and Resurreccion, 2009; Arrutia et al., 2020). However, there are many limitations when PNM is used as a poultry feed ingredient, including the imbalanced amino acid profiles, anti-nutritional factors (the phytate content is about 1.5%), and vulnerability to contamination by pathogenic bacterias and mycotoxin (Gupta,

1987; Batal et al., 2005; Tola and Kebede, 2016). These factors seriously limit the use of PNM as a high-quality protein raw material in poultry feed.

Scientists have expended much effort to make improvements and increase utilization of PNM, such as by supplementing essential amino acids (usually Lys and Met), enzymolysis and fermentation (Driggers and Tarver, 1958; Shi et al., 2014; Yang et al., 2016a). Solid-state fermentation is a biological treatment technology that has a long application history in the food and feed industries. The process involves microorganisms growing on solid materials under controlled conditions, in the absence of free water, the water required for microbial growth and metabolism is in an absorbed state within the solid matrix (Olukomaiya et al., 2019). Many studies have described how solid-state fermentation can improve the nutritional value of plant protein sources and show good prospects for reducing anti-nutritional factor levels and promoting nutrient utilization (Hirabayashi et al., 1998; Hu et al., 2016; Li et al., 2022b). It is also a promising approach to improving the nutrient availability of PNM by solid-state fermentation, which significantly improves the crude protein content, acid-soluble oligopeptide content and in vitro digestibility (Yang et al., 2016a). However, there are few comments about the potential of fermented peanut meal (FPNM) as a feed ingredient to formulate poultry diets. This article briefly describes the current application and limitations of PNM as an ingredient in poultry feed and introduces the nutritional value enhancement of other plant protein sources through solid-state fermentation. The purpose is to analyze the feasibility of solid-state fermentation in improving the nutritional value of PNM as poultry feed.

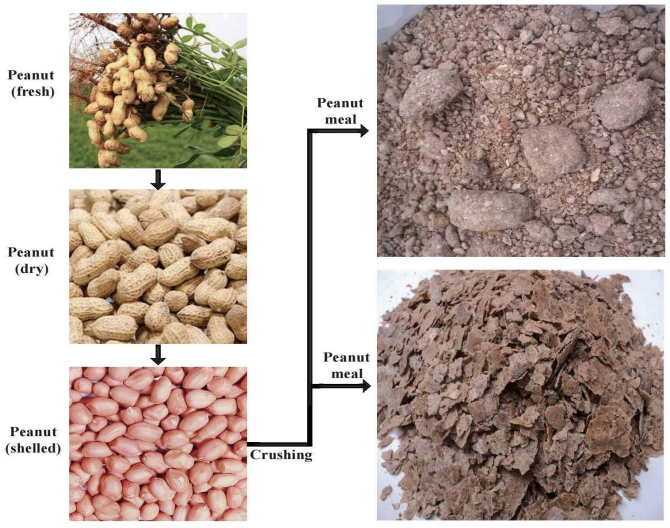

Figure 1. The production process and two main morphological forms of PNM

3. The application of PNM as poultry feed

Early attempts at using PNM as a source of protein for poultry indicated that its nutritional value was inferior to that of soybean meal. Coupled with its limited yield at that time, a general addition to the diets of broilers in the United States was 3–4% (Batal et al., 2005). As it may be gathered from Table 1, the effects of PNM on the production performance of poultry vary in different reports. As early as 1946, Heuser et al., (1946) reported that the use of PNM and soybean meal in the diets of broilers at a ratio of 1:1 would not affect their performance. The recent study by Saleh et al., (2022) also found that the growth performance and protein digestibility of broilers were not affected when 5% or 10% PNM was added to corn-soybean meal-based diets. However, a previous study found that when PNM replaced 50% of soybean meal, the growth performance of broilers was reduced (Douglas and Harms, 1959). el Boushy and Raterink, (1989) reported that when the proportion of PNM in the diet increased from 5% to 15%, the growth performance and feed conversion of broilers decreased even if the Lys and Met in the diet were sufficient . Costa et al., (2001a) also observed that, under the premise of meeting the minimum amino acid requirements of broilers, increasing the addition ratio of PNM (0%, 10%, 20% and 32%) showed a decreasing trend in growth performance.

In terms of application to laying hens, Pesti et al., (2003) found that PNM addition at 21.4–35.5% would reduce the egg weight of 22–28-week laying hens compared to corn-soybean meal-based diets. Research on egg ducks obtained similar results: replacing soybean meal with PNM at a ratio of 100% had a negative effect on feed intake, feed conversion and egg weight (Xia et al., 2022a). These conflicting results may be due to differences in the production process, chemical composition, levels of PNM addition and anti-nutritional factor of PNM. What is certain is that different sources of PNM directly affect the application effect in broilers. Studies have shown that when feeding broilers with high-oleic acid PNM, their growth performance and breast meat yield are lower than on corn-soybean meal-based diets, but the content of unsaturated fatty acids in breast muscle is significantly increased (Toomer et al., 2020a, 2020b). A clear understanding of the limiting factors for the use of PNM in poultry is therefore essential.

Table 1. List of the effects of PNM on the production performance of poultry

Years	Species		Control diet	PNM proportion	Production performance	Other effects	Reference
1946	Broilers	Comb White Leghorn broilers	Corn-soybean	50% substitute soybean meal	No differences	—	Heuser et al.
1959	Broilers	Vantress × White Plymouth broilers	Corn-soybean	50% substitute soybean meal	Decreased BW	—	Douglas and Harms
1989	Broilers	Hybro broilers	Corn-soybean	10% of diet	Decreased BW	—	EL Boushy and Raterink
1989	Broilers	Hybro broilers	Corn-soybean	15% of diet	Decreased BW	—	EL Boushy and Raterink
2001	Broilers	Ross 208 broilers	Corn-soybean	10% of diet	No differences	—	Costa et al.
2001	Broilers	Ross 208 broilers	Corn-soybean	20% of diet	No differences	—	Costa et al.
2001	Broilers	Ross 208 broilers	Corn-soybean	32% of diet	Decreased BW; increased F:G ratio	—	Costa et al.
2009	Broilers	Vencob broilers	Corn-soybean	25% substitute soybean meal	Increased BW	—	Ghadge et al.
2009	Broilers	Vencob broilers	Corn-soybean	50% substitute soybean meal	Increased BW; decreased F:G ratio	—	Ghadge et al.
2009	Broilers	Vencob broilers	Corn-soybean	75% substitute soybean meal	Increased BW; decreased F:G ratio	—	Ghadge et al.

2009	Broilers	Vencob broilers	Corn-soybean	100% substitute soybean meal	Increased BW; decreased F:G ratio	—	Ghadge et al.
2016	Broilers	Lohman broilers	Corn-soybean	50% substitute soybean meal	No differences	—	Ata
2016	Broilers	Lohman broilers	Corn-soybean	100% substitute soybean meal	Increased BW	—	Ata
2020	Broilers	Ross 708 broilers	Corn-soybean	12% of diet	Decreased BW, carcass and breast meat yields	Increased PUFA in breast meat	Toomer et al.
2022	Broilers	Cobb 500 broilers	Corn-soybean	10% of diet	No differences	Decreased serum TC, TG and LDL-cholesterol	Saleh et al.
2003	Hens	Hyline W-36 White Leghorn hens	Corn-soybean	3.8 g/hen per d	Decreased egg weight (first 6 weeks)	—	Pesti et al.
2013	Hens	Rugao laying hens	Corn-soybean	5.3% substitute soybean meal	No differences	Decreased egg yolk cholesterol content	Lu et al.
2013	Hens	Rugao laying hens	Corn-soybean	10.6% substitute soybean meal	No differences	Decreased egg yolk cholesterol content	Lu et al.
2013	Hens	Rugao laying hens	Corn-soybean	15.9% substitute soybean meal	No differences	—	Lu et al.
2022	Ducks	Longyan laying ducks	Corn-soybean	25% substitute soybean meal	No differences	Decreased serum GSH	Xia et al.
2022	Ducks	Longyan laying ducks	Corn-soybean	50% substitute soybean meal	Decreased feed intake	Decreased serum GSH	Xia et al.

2022	Ducks	Longyan laying ducks	Corn-soybean	75% substitute soybean meal	Decreased feed intake	Decreased serum GSH	Xia et al.
2022	Ducks	Longyan laying ducks	Corn-soybean	100% substitute soybean meal	Decreased FI and egg weight; increased F:G ratio	Decreased serum GSH; increased serum MDA	Xia et al.

Abbreviations: BW, body weight; F:G ratio, feed intake: weight gain; FI, feed intake; PUFA, polyunsaturated fatty acids; TC, total cholesterol; TG, triglycerides; GSH, glutathione; MDA, malonaldehyde. "—": no relevant content.

4. The limiting factors of PNM as poultry feed

The factors limiting PNM as a poultry feed ingredient can be summarized as nutritional and non-nutritional issues.

4.1 Imbalance of amino acid composition

The imbalance in amino acid composition is one of the nutritional limitations. Table 2 shows the contents of amino acids in PNM and soybean meal reported in various literature. Obviously, although there are some slight differences in the content of amino acids in PNM from different resources, their trends are similar. Compared with amino acids in soybean meal, Arg, Glu and Gly in PNM are relatively high, while essential amino acids such as Met, Lys, Thr, Ile and Val are relatively low. In addition, the ratio of Arg to Lys in PNM is 3.27–3.55, far exceeding the recommended range of 1.10–1.18 proposed by the National Research Council Nutrient Requirements of Poultry (Council, 1994). Studies have shown that excessive Arg can inhibit the absorption of Lys, leading to a further deficiency of Lys in the body (Zampiga et al., 2018). Driggers and Tarver (1958) found that by adding Lys to broiler diets, PNM could replace 50% of the soybean meal. Shortly afterwards, researchers demonstrated that Lys was the first limiting amino acid in the corn-PNM-based diet, followed by Met and Thr (Douglas and Harms, 1959; Waldroup and Harms, 1963). Robbins (1987) further discovered that the content of Cys was low in PNM and established a model of Cys deficiency in broilers by supplementing other amino acids in a corn starch-PNM-based diet. In addition, due to the need for heat pressing before solvent extraction of peanut oil, it is possible that overprocessing may reduce the protein quality of PNM. Zhang and Parsons (1996) reported that the amino acid digestibility decreased with the prolongation of the heat pressing time, especially the digestibility of Met, which decreased from 87% to 57%. As described above, the focus must be placed on the balance of amino acid composition, especially the concentration and proportion of Lys, Met, Thr and Cys when formulating poultry diets with PNM.

Table 2. The contents of amino acids in PNM and soybean meal reported in various literature

Items	PNM		Soybean Meal	
	USA (Zhang and Parsons, 1996)	**China** (Chinese Feed Database News Web Center., 2020)	**New Zealand** (Cowieson et al., 2020)	**China** (Chinese Feed Database News Web Center., 2020)
CP, %	47.8	47.8	48.2	47.9
Essential amino acids				
Met	0.47	0.41	0.66	0.68
Lys	1.66	1.40	2.98	2.99
Thr	1.31	1.11	2.02	1.85

Arg	5.90	4.88	3.68	3.43
Ile	1.60	1.25	2.11	2.10
Leu	3.10	2.50	3.52	3.57
Val	1.91	1.36	2.32	2.26
His	1.10	0.88	1.22	1.22
Phe	2.35	1.92	2.38	2.33
Non-essential amino acid				
Gly	2.88	—	2.17	—
Ser	2.56	—	2.72	—
Pro	2.12	—	2.37	—
Ala	1.91	—	2.11	—
Asp	6.36	—	5.91	—
Glu	10.15	—	8.44	—
Cys	0.69	0.40	0.65	0.73
Tyr	1.42	1.39	1.47	1.57

"—": not determined.

4.2 Phytic acid

Myo-inositol 1, 2, 3, 4, 5, 6-hexakisphosphate (IP6), also known as phytic acid, has an asymmetrical six-carbon ring structure (Figure 2A) (Cangussu et al., 2018). As the major anti-nutritional factor in PNM (content is about 1.5%), phytic acid strongly binds to protein molecules by phosphate groups, significantly reducing the digestibility and utilization of protein (Nyman and Bjorck, 1989). In addition, phytate also binds to the basic amino acid residues of enzymes, which in turn inhibits the activities of trypsin, amylase and pepsin, ultimately affecting the digestion and absorption of nutrients (Ren et al., 2017). Cowieson et al., (2007) believed that phytic acid increased the excretion of endogenous nitrogen in animals, resulting in nitrogen loss in the body and reducing the utilization of protein by animals. Moreover, phytic acid can firmly adhere to zinc, copper, calcium, magnesium, iron and other positively charged metal ions in the gastrointestinal tract of animals to form insoluble phytic acid complexes, resulting in reduced bioavailability of trace mineral elements (Hunt and Vanderpool, 2001). Microbial phytase, which belongs to the hydrolase family, can be produced by fungi or bacteria. The degradation of phytic acid is carried out by catalyzing the hydrolysis of phytic acid into low-grade myo-inositol phosphate and inorganic phosphorus (Figure 2B) (Bohn et al., 2008; Gupta et al., 2015). Almost no endogenous phytase exists in the digestive tract of poultry, so it is difficult for the body to decompose phytic acid. The benefits of supplemental phytase in poultry diets are clearly established, including improved nutrient utilization, growth performance, bone mineralization and so on (Walk et al., 2012; Babatunde et al., 2019, 2020). Regarding the application effect of phytase in the PNM diet, Driver et al., (2006) proved that adding phytase to a corn-PNM diet increased the nitrogen-corrected apparent metabolizable energy of broilers from 3209 kcal/kg to

3559 kcal/kg.

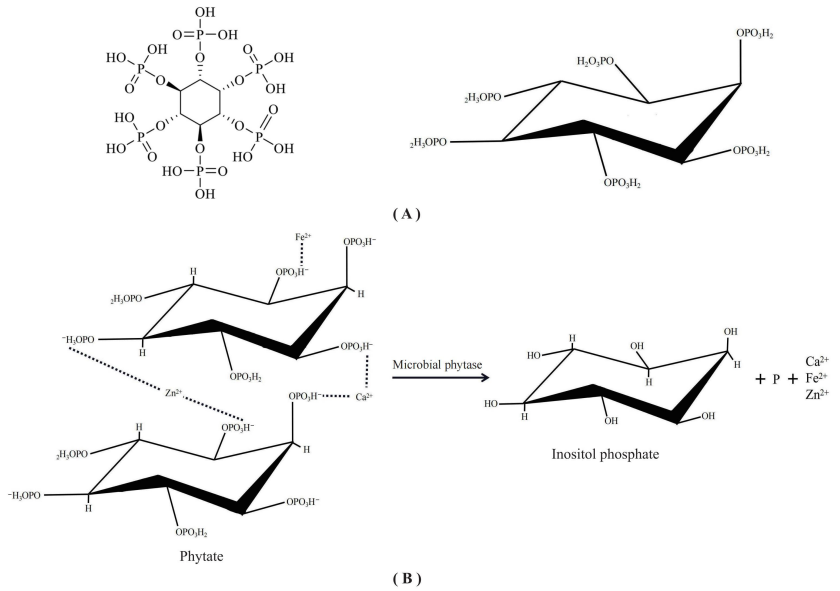

Figure 2. The mechanism of phytase to hydrolyze phytate. (A) Phytic acid; (B) The mechanism of microbial phytase action

4.3 Risk of aflatoxin pollution

Aflatoxin is a toxic metabolite produced by a number of fungi, including *Aspergillus parasiticus* and *Aspergillus flavus*. The earliest report on aflatoxins in PNM dates back to 1960, when an outbreak of "X" disease in turkeys occurred in the UK, resulting in the death of approximately 100,000 birds due to the ingestion of PNM contaminated with aflatoxins (Stevens, A. J., Saunders, C. N., Spence, J. B., Newham, 1960). Since then, aflatoxins contamination in PNM has gradually become an increasing concern around the global feed industry. Currently, twenty kinds of aflatoxins have been isolated, such as B_1, B_2, M_1, M_2, G_1 and G_2, with similar structures, of which aflatoxin B_1 (AFB_1) is the most common and carcinogenic one. Kana et al., (2013) investigated the contamination of aflatoxins in PNM in Cameroon; the results revealed that the positive rate was 100% and the total aflatoxin ($B_1 + B_2 + G_1 + G_2$) concentrations ranged from 39 to 950 μg/kg. (Chen et al., 2013) collected 322 samples of peanut flour from Taiwan, and the detection results showed that the positive rates for AFB_1, AFB_2 and AFG_2 were 100%, 89.6% and 8.3%, respectively. The average concentrations of AFB_1, AFB_2 and AFG_2 were 7.02 μg/kg, 1.54 μg/kg and 0.21 μg/kg, respectively, and AFG_1 was not detected. It can be seen that PNM was widely contaminated with aflatoxins, especially AFB_1. In fact, the accumulation of aflatoxins in the PNM is not accidental, as most peanuts have already been infected with *Aspergillus parasiticus* or *Aspergillus flavus* during the growth process. Moreover, PNM is an excellent culture medium for fungi, and the release of aflatoxins increases with

the proliferation of fungi (Yu et al., 2020a). During oil extraction, small portions of aflatoxins in peanuts are transferred to the oil phase, and most of them remain in the PNM (Arias et al., 2018). When poultry are fed diets containing AFB_1, it will be absorbed by the small intestine and bind to plasma albumin. However, it is not AFB_1 itself that is toxic to the host, but its enzymatic transformation products, including AFB_1-8,9-exo-epoxide, AFM_1, AFQ_1 and AFP_1 (Taguchi et al., 2016). Studies have shown that AFB_1-8,9-exo-epoxide is critically important in the acute and chronic toxicity of AFB_1, which can interfere with protein synthesis and lead to cell death, oxidative damage, decreased productivity and even death (Jiang et al., 2015; Rajput et al., 2019). Furthermore, due to the lipophilicity of AFB_1, it can be absorbed by the intestine and distributed to the liver, muscle, kidney, and fat tissues, posing further threats to human food safety (Bedoya-Serna et al., 2018). Previous studies revealed that, when the concentration of AFB_1 in the broiler diet exceeded 1800 μg/kg, 3.84 μg/kg AFB_1 was detected in the liver in the second week (Fowler et al., 2015). Therefore, when PNM is added to the poultry diet, the mycotoxin content must be monitored as a key variable, and consideration should be given to using mycotoxin adsorbents or binders to control toxin levels (Xia et al., 2022a). The European Commission has established the most stringent regulations for mycotoxins in feed, with the maximum allowable total AFs (AFB_1 + AFB_2 + AFG_1 + AFG_2) set at 10 μg/kg and 5.0 μg/kg of AFB_1 (European Commission., 2010). In China, based on the Chinese Feed Hygiene Standard (2017 version), the maximum allowable limit of PNM as a feed ingredient is 50 μg/kg, and the maximum allowable limit in broiler feed is 10 μg/kg (General Administration of Quality Supervision., 2017). Reassuringly, the risk of aflatoxin contamination can be effectively controlled by planting resistant peanut varieties combined with necessary crop management (Yu et al., 2020a). Through a series of policy formulation and implementation efforts, the contamination of peanuts with AFs has improved (Ding et al., 2012).

Many physical, chemical and biological methods have been verified to be able to inactivate or detoxify the aflatoxins in contaminated feedstuffs. Among them, the biological method is a very promising measure due to its specificity, irreversibility and efficiency in detoxification (Food and Agriculture Organization of the United Nations (FAO)., 2001). Some specific microorganisms can play a role in the adsorption and degradation of AFB_1. The physical adsorption of AFB_1 by lactic acid bacteria is related to the cell wall, which is a rapid and reversible process, and the adsorption capacity varies with different bacterial species and doses (Bueno et al., 2007). In addition, AFB_1 can also be degraded by extracellular enzymes secreted by microorganisms, such as *Bacillus* spp. or white rot fungi (Alberts et al., 2009; Gao et al., 2011; Xu et al., 2017). According to the structure analysis of the degradation products, the epoxide ring, carbonyl and unsaturated carbon-carbon double bonds of AFB_1 are generally attacked, forming water-soluble salts without toxicity (Motomura et al., 2003).

4.4 Other limiting factors

PNM can provide good sources of carbon, nitrogen and energy for bacterial growth and reproduction, which makes it prone to contamination by pathogenic

bacteria such as *Salmonella* and *Escherichia coli* during storage. Exposure to pathogenic bacteria poses numerous and diverse threats to developing animals (Brock et al., 2014). In addition, PNM contains large amounts of highly unsaturated fatty acids, especially linoleic and linolenic acids, which are oxidized to produce free radicals and peroxynitrite. This makes PNM prone to oxidation deterioration, resulting in decreased shelf life, poor palatability and nutritional losses (Talcott et al., 2005). These are also a limiting factor for the use of PNM as a feed ingredient for poultry.

5. Solid-state fermented plant protein sources in the diets of poultry

In general, the production process of plant protein sources mainly consists of pretreatment, microbe inoculation, fermentation procedure and product collection (Figure 3). It is worth noting that there will be one-step fermentation or multi-step fermentation according to the raw materials and purposes (Yang et al., 2016a; Olukomaiya et al., 2019). It has been shown that many beneficial compounds have been proven to be produced by fermentation, such as enzymes, organic acids, flavor compounds and bioactive substances. Table 3 summarizes the nutritional changes of common plant protein sources during fermentation and their application effects in poultry.

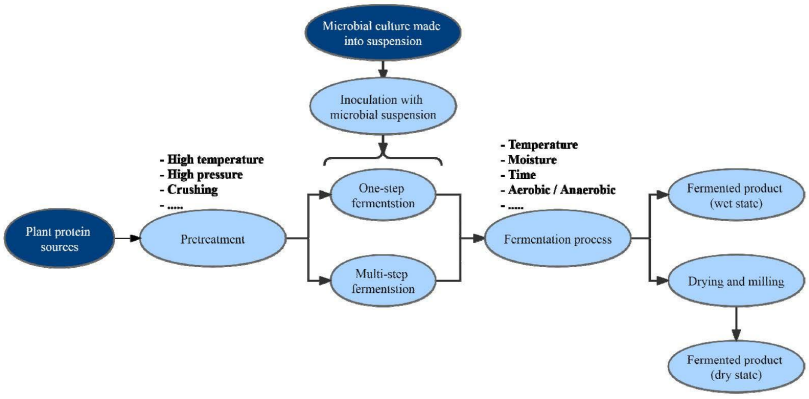

Figure 3. Schematic representation of steps involved in the solid-state fermentation of plant protein sources

Proteins in animals are digested and decomposed into various kinds of amino acids and peptides, which are then absorbed by animals (Couri et al., 2000; Wang et al., 2019; Lu et al., 2023). The modern protein nutrition theory holds that only part of the proteins are absorbed by animals in the form of amino acids, while most of the proteins are absorbed in the form of peptides. The peptide transport system has the characteristics of fast speed, low energy consumption and non-saturation of transport carriers (Rérat, 1995). Therefore, the peptide content is an important index for evaluating the fermentation products of plant protein

sources. Trichloroacetic-acid-soluble protein (TCA-SP) is usually used as an indicator for plant protein source fermentation, which consists of peptides with a molecular weight less than 10 kDa, free amino acids and small amounts of non-protein nitrogen compounds (Li et al., 2019, 2022b). Other evaluation indices include the degree of degradation of anti-nutritional factors or the increase in organic acids (generally lactic acid). Next, we will briefly review the related research on the solid-state fermentation of soybean meal, rapeseed meal and cottonseed meal, as they are similar to PNM in terms of nutritional composition, in the hope of providing some basic guidance on the PNM fermentation.

Table 3. The nutritional changes of common plant protein sources during fermentation and their application effects in poultry

Years	Substrates	Microorganisms	Animals applied	Nutritional improvement / beneficial effects	Reference
1998	Soybean meal	*Aspergillus usamii*	Broilers	Decreased phytic acid phosphorus (complete degradation). Fermented soybean meal improved BW, retained phosphorus and femoral phosphorus in broilers	Hirabayashi et al.
2006	Soybean meal	*Aspergillus niger*	Broilers	Fermented soybean meal improved BW, ileum villi length and width in broilers	Mathivanan et al.
2007	Soybean meal	*Aspergillus oryzae*	Broilers	Fermented soybean meal improved ADG, FI, serum phosphorus, IgM and IgA; decreased serum urea nitrogen in broilers	Feng et al.
2016	Soybean meal	*Bacillus amyloliquefaciens*	—	Decreased trypsin inhibitor, raffinose and stachyose; increased antioxidant activity and metal-chelating ability	Chi and Cho
2016	Soybean meal	*Bacillus subtilis*	—	Decreased trypsin inhibitor and β-conglycinin	Seo and Cho
2020& 2022	Soybean meal	*Bacillus amyloliquefaciens, Lactobacillus acidophilus* and *Saccharomyces cerevisiae*	Broilers	Decreased glycinin and β-conglycinin; increased CP and TCA-SP. Fermented soybean meal improved energy digestibility and SID of amino acids in broilers	Li et al.
2023	Soybean meal	*Bacillus* spp. yeast, *Lactobacillus* spp. and *Clostridium* spp.	Laying hens	Increased the CP, amino acids and organic acids; decreased NDF and ADF. Fermented soybean meal improved the laying performance, egg quality, intestinal barrier function and follicle development in hens	Lu et al.
2001	Rapeseed	*Rhizopus oligosporus*	—	Increased nitrogen and protein contents; decreased	Vig and Walia

	meal			glucosinolates, thiooxazolidones, phytic acid and CF	
2016	Rapeseed meal	*Bacillus subtilis, Candida utilis* and *Enterococcus faecalis*	Broilers	Increase CP and small peptides; decreased CF, glucosinolate, isothiocyanate, tannin and phytic acid. Fermented rapeseed meal improved antioxidant level and intestinal morphology of broilers	Hu et al.
2017	Rapeseed meal	*Lactobacillus acidophilus, Bacillus subtilis,* and *Aspergillus niger*	Broilers	Fermented rapeseed meal decreased colonization of *Salmonela* and *Typhimurium*; improved growth performance	Ashayerizadeh et al.
2019	Rapeseed meal	*Bacillus licheniformis,* Yeast and *Lactobacillus*	Broilers	Improved the sensory properties, CP, lactic acid and total amino acid; decreased glucosinolate and NDF. Fermented rapeseed meal improved productivity performances of broilers	Wang et al.
2022	Rapeseed meal	*Bacillus subtilis*	Japanese quail	No significant differences were found	Wengerska et al.
2022	Rapeseed meal	*Bacillus subtilis* and *Aspergillus niger*	Laying hens	Increased lactic acid bacteria and CP; decreased pH, dry matter, CF and anti-nutritional factors. Fermented rapeseed meal improved egg production and egg mass in hens	Taheri et al.
2012	Cottonseed meal	*Bacillus subtilis*	Broilers	Decreased free gossypol. Fermented cottonseed meal improved growth performance and immunity in broilers	Tang et al.
2017	Cottonseed meal	*Bacillus subtilis, Aspergillus niger* and *Aspergillus oryzae*	Broilers	Decreased CF and free gossypol; increased CP and lactic acid bacteria. Fermented cottonseed meal improved intestinal development and growth performance; decreased abdominal fat yield in broilers	Jazi et al.
2019	Cottonseed meal	*Candida tropicalis*	Broilers	Fermented cottonseed meal decreased abdominal fat yield and subcutaneous fat thickness in broilers	Niu et al.

5.1 Soybean meal

Soybean meal is the most widely used feedstuff as a plant protein source. The anti-nutritional factors, such as protease inhibitors, soybean agglutinin, antigen protein, phytic acid and soybean oligosaccharides in soybeans, are difficult to remove in the process of oil extraction by heating, pressing and leaching, so they are largely left in the soybean meal (Domagalski et al., 1992). However, most of these anti-nutritional factors can be degraded by solid-state fermentation. Hirabayashi et al., (1998) found that fermentation of soybean meal with *Aspergillus usamii* can efficiently degrade the phytic acid and provide more available phosphorus. Trypsin inhibitor is a protein-based anti-nutritional factor in soybean meal that inhibits the activity of trypsin and chymotrypsin (Liener, 1994). Reports from Chi and Cho, (2016); Seo and Cho, (2016) showed that fermented by *Bacillus* spp. can effectively degrade this component in soybean meal. Furthermore, Wang et al., (2020) achieved a 94.2% degradation efficiency of trypsin inhibitor by using *Bacillus subtilis* to conduct two-stage solid-state fermentation of soybean meal. At the same time, the contents of soybean globulin and β-conglycinin, which are antigens that trigger hypersensitivity reactions, were also effectively reduced. Chi and Cho, (2016) also reported that raffinose and stachyose, as indigestible oligosaccharides in soybean meal, were almost completely degraded after fermentation by *Bacillus amyloliquefaciens*. Solid-state fermentation can also optimize the composition of nutrients in soybean meal. (Hong et al., 2004) showed that after fermentation by *Aspergillus oryzae*, the amount of macromolecular protein content (> 20 kDa) in soybean meal decreased from 76.9% to 17.7%. In addition, Li et al., (2022b) reported that the macromolecular proteins in soybean meal were degraded into small peptides and free amino acids by *Bacillus amyloliquefaciens* fermentation, the total amino acid content increased by 2.56%, and the crude fiber decreased by 7.56%. They also found that the standardized ileal digestibility of amino acids in broilers was significantly increased. In vivo tests showed that dietary fermented soybean meal supplementation had beneficial effects on improving growth performance, promoting intestinal development, reducing the colonization rate of pathogenic bacteria in the intestine, and enhancing the immune responses of poultry; the effects on laying birds included improving laying performance, egg quality and follicle development (Feng et al., 2007; Kim et al., 2016; Jazi et al., 2019; Lu et al., 2023).

5.2 Rapeseed meal

The crude protein content of rapeseed meal is 35–40%, and the annual production is more than 10 million tons in China, making it a potential substitute for soybean meal in poultry feed (Wang et al., 2019; Wu et al., 2020). However, the application of rapeseed meal is restricted due to its poor palatability and anti-nutritional factors such as glucosinolates, phytic acid and tannin, especially the high content of glucosinolates, which can cause thyroid enlargement, growth inhibition and a decrease in production (Tripathi and Mishra, 2007). Early research showed that solid-state fermentation of rapeseed meal with *Rhizopus oligosporus* reduced the content of glucosinolates, phytic acid and crude fiber by

43.1%, 42.4% and 25.5%, respectively, without loss of essential nutrients (Pal Vig and Walia, 2001). Subsequent reports have shown that fermentation of rapeseed meal with mixed microorganisms (*Bacillus licheniformis*, *Yeast* and *Lactobacillus*) can degrade most rapeseed meal proteins into small peptides with molecular weights less than 9.5 kDa, which significantly improves the sensory properties and bioavailability of rapeseed meal (Wang et al., 2019). In the study of the fermentation of rapeseed meal with lactic acid bacteria, an increase in organic acid content was also found (Ashayerizadeh et al., 2017; Wu et al., 2020). Xu et al., (2012); Taheri et al., (2022) reported that dietary fermented rapeseed meal supplementation had beneficial effects on the growth performance and egg quality of poultry. These changes indicate increased nutrient utilization of the rapeseed meal. There is evidence suggesting that fermented rapeseed meal had a higher apparent metabolizable energy value and standardized ileal digestibility of amino acids compared to unfermented rapeseed meal in broilers (Wu et al., 2020). Moreover, Ashayerizadeh et al., (2017) found that fermented rapeseed meal could effectively reduce the colonization of *Salmonella Typhimurium* in broilers and also showed potential as an anti-stress product.

5.3 Cottonseed meal

Cottonseed meal is a byproduct of cottonseed oil extraction and is also considered to be a potential source of plant protein. However, its application in poultry diets is greatly limited by the presence of gossypol (He et al., 2015). Solid-state fermentation is recognized as an effective method for reducing gossypol (Tang et al., 2012; Jazi et al., 2017a). Additionally, the metabolites such as vitamins and organic acids produced in the fermentation process can improve the nutritional value of cottonseed meal (Jazi et al., 2017a). Previous research has shown that fermented cottonseed meal has positive effects on the antioxidant capacity, intestinal development, growth performance and immune function of poultry (Tang et al., 2012). Niu et al., (2019) further demonstrated that fermented cottonseed meal can reduce abdominal fat content and subcutaneous fat thickness in broilers by regulating the metabolism of organic acids, fatty acids and amino acids.

6. Improving the nutritional value of PNM through solid-state fermentation

According to the previous description, we aim to achieve the following objectives by solid-state fermentation of PNM: (1) increase the application ratio of PNM in poultry feed while reducing the supplementation of commercial amino acids; (2) reduce the content of crude fiber and phytic acid in PNM; (3) reduce the contamination of pathogenic microorganisms in PNM during storage; and (4) obtain metabolites or bioactive substances with beneficial effects on poultry health. According to the nutritional characteristics of PNM and the reports of other plant protein sources, we believe that the expected objectives can be achieved by certain technical means.

6.1 Bio-transformation and bio-Conversion of PNM

PNM can provide good nutritional and environmental conditions for the

reproduction of bacteria as a solid-state medium. Currently, there are few reports about the solid-state fermentation of PNM, but some research on the bio-modification or bio-conversion of PNM may provide some insights. Table 4 summarizes the related reports. PNM is a rich carbon source and can be used as a substrate to produce high-value products such as rhamnolipids through bio-conversion (Zhao et al., 2023). Using nitrogen sources from PNM to produce D-lactic acid and succinic acid is considered to be a more economical and efficient method (Wang et al., 2011a; Shen et al., 2015). In addition, many reports have shown that the conversion of peanut proteins into bioactive peptides is mainly achieved by hydrolysis or fermentation methods and that the resulting products have excellent antioxidant, immunomodulation, antimicrobial and anti-cancer properties (Zhang et al., 2014; Hariharan et al., 2023). Regarding the molecular weight size of peptides, 1–10 kDa had the highest antioxidant activity (Su et al., 2011; Wei et al., 2012). Wang et al., (2011b) fermented PNM with lactic acid bacteria and also found an increase in antioxidant activity, but they suggested that the mechanism might be related to the transformation of rutin in PNM to quercetin. Yang et al., (2016a) attempted to improve the nutritional and functional properties of PNM by liquid-state fermentation (80% moisture), and the results showed that the content of crude protein, acid-soluble oligopeptides, organic acids and in vitro digestibility of PNM were significantly improved after fermentation, and the content of amino acids was balanced by fermentation. In future studies, we hope to achieve the same effect through solid-state fermentation.

6.2 Selection of strains for solid-state fermentation of PNM

The screening of fermentation strains must comply with the principle of safety, and the selected strains cannot destroy the inherent balance of the ecological environment or produce toxic and harmful substances. In addition, strains for fermenting PNM must have the potential to decompose proteins and cellulose, degrade phytic acid and inhibit pathogenic microbial pollution, which requires a specific screening process to select the strains.

Bacteria and fungi are commonly used in the fermentation of plant protein sources. Studies have shown that bacteria usually have genes coding for plant cell wall-degrading enzymes and are more easily capable of utilizing cellulose as a carbon source than fungi, which is beneficial for the decomposition of fiber in PNM. In addition, bacteria are more suitable for industrial applications than fungi due to their advantages, such as fast growth rates, good resistance and ease of operation (Štursová et al., 2012). *Bacillus* spp. are famous for their production of various metabolic products, such as cellulase, amylase, lipase, vitamins and antimicrobial peptides, and especially for their important role in degrading peanut proteins. After entering the intestine, *Bacillus* spp. will consume a large amount of oxygen to maintain the anaerobic environment of the intestine, thus inhibiting the growth of aerobic pathogenic bacteria and ultimately maintaining the balance of microflora in the animal intestine (Yang et al., 2016a). Lactic acid bacteria are generally regarded as safe strains (GRAS), so they are often used for health-promoting purposes as probiotics, as well as widely used in the fermentation of plant protein sources (Teusink and Smid, 2006). During the

fermentation process, organic acids are produced to reduce the pH of the feed and inhibit the growth of pathogenic bacteria (Li et al., 2023b). Forestier et al., (2001) found that lactic acid bacteria can inhibit the adhesion of pathogenic bacteria such as *Salmonella* and *Escherichia coli* to intestinal cells, modulate the immune response and protect the intestinal barrier.

Table 4. Bio-transformation and bio-conversion of PNM

Years	Preparation method	Strain / enzyme	Objective	Characteristic	Reference
2016	Fermentation	*Bacillus licheniformis*	Enhancement of nutritional and antioxidant properties	The nutritional properties and antioxidant capacity of PNM were enhanced	Yang et al.
2011	Fermentation	*Bifidobacterium longum* *Lactobacillus casei* *Lactobacillus acidophilus* *Lactobacillus plantarum*	Produce fermented peanut flour	Antioxidant activity was increased	Wang et al.
2012	Fermentation	*Aspergillus oryzae* *Aspergillus niger*	Produce antioxidant peptides	Peptide fraction of 3–10 kDa showed the highest antioxidant activity	Wei et al.
2013	Fermentation	*Bacillus subtilis*	Produce antioxidant peptides	High antioxidant peptide activity was obtained	Zhang et al.
2015	Fermentation	*Actinobacillus succinogenes*	Produce succinic acid	PNM can be used as an efficient and economical source of nitrogen	Shen et al.
2023	Fermentation	*Pseudomonas aeruginosa*	Produce rhamnolipid	Produced rhamnolipid exhibited good physicochemical and antimicrobial activities	Zhao et al.
2010	Hydrolysis	*Sporolactobacillus* sp.	Produce D-lactate	High D-lactate production	Wang et al.
2011	Hydrolysis	Crude enzyme obtained from *Aspergillus oryzae*	Produce antioxidant peptides	Peptide fraction of 1–3 kDa showed the highest antioxidant activity	Su et al. (Su et al., 2011)
2013	Hydrolysis	Alcalase from *Bacillus licheniformis*	Produce bioactive peptides	Bioactive peptides have a potential benefit for blood pressure regulation	White et al. (White et al., 2013)

7. Conclusions and perspectives

As reported in this review, enhancing the nutritional value of PNM through solid-state fermentation is technically challenging but feasible. It should be noted that further studies should primarily focus on improving amino acid imbalance, reducing phytic acid content and preventing microbial contamination. Obviously, the quality of the final fermented product is closely related to the fermentation microorganisms used. This suggests that we need to screen microorganisms based on the characteristics of PNM and ensure their biosafety. We expect that after solid-state fermentation, PNM will have a higher nutrient availability in poultry and an enhanced shelf life, while not excluding some enhanced functional characteristics, such as antioxidant capacity.

Chapter III

Objectives and thesis structure

Chapter III. Objectives and outline of the thesis

1. Objective

In our study, we aimed

(1) Design specific screening processes based on the nutritional characteristics of peanut meal (PNM) to identify two bacterial strains suitable for fermentation. One strain should demonstrate a high potential for extracellular secretion of complex enzymes, including proteases, cellulases, and phytases. The other strain should exhibit efficient acid production, antimicrobial activity, and antioxidative properties.

(2) Once the target bacteria have been screened, we will employ conventional techniques to conduct solid-state fermentation of PNM. The primary focus will be on investigating the changes in chemical indicators (such as crude protein, Trichloroacetic-acid-soluble protein, crude fiber, phytic acid, lactic acid) and physical properties of PNM after fermentation. Additionally, we will further evaluate the digestibility of amino acids and metabolic energy values of fermented peanut meal (FPNM) in broiler chickens.

(3) Based on the determined chemical composition of FPNM and PNM, as well as their metabolizable energy in broilers, we will investigate the optimal supplementation ratio of FPNM in broiler diets and assess their effects on growth performance and other relevant aspects.

2. Outline of the thesis

For the purpose of improving the nutritional value of PNM by solid-state fermentation, we provide an action framework and strategies (Figure 4). The following steps were designed and completed in sequence:

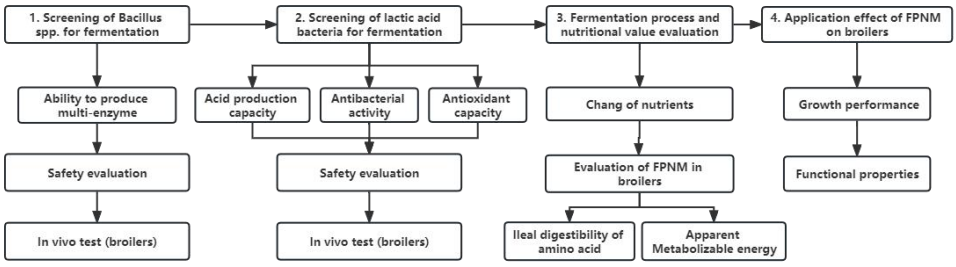

Figure 4. The technical route of the solid-state fermentation process of PNM and its application in broilers

(1) Screening and characterization of *Bacillus* spp. based on the ability to produce multi-enzymes, including protease, cellulase and phytase. It should be noted that the screened strains need to be comprehensively tested to ensure that

they do not have adverse biological characteristics, such as harmful biochemical effects, antibiotic resistance and virulence factors. (**Chapter IV**)

Reference: Chong Li†, Shuzhen Li†, … Yves Beckers and Huiyi Cai. Screening and characterization of *Bacillus velezensis* LB-Y-1 toward selection as a potential probiotic for poultry with multi-enzyme production property [J]. *Frontiers in Microbiology.* 2023, 17(4): 1143265.

(2) Screening and characterization of lactic acid bacteria based on acid production capacity, antibacterial activity against selected pathogens and antioxidant capacity. (**Chapter V**)

Reference:Chong Li†, Shaolong Wang†, … Yves Beckers and Huiyi Cai. Screening and characterization of *Pediococcus acidilactici* LC-9-1 toward selection as a potential probiotic for poultry with antibacterial and antioxidative properties [J]. *Antioxidants*, 2023, 12(2): 215.

(3) The fermentation and nutritional value evaluation of PNM in broilers. Specifically, analysis of the changes in the nutritional composition of PNM after solid-state fermentation and determination of the effect of feeding broilers a diet of PNM and FPNM on ileal amino acid digestibility values and apparent metabolizable energy. (**Chapter VI**)

Reference: Shuzhen Li†, **Chong Li**†, … Huiyi Cai and Guohua Liu. Effects of solid-state fermentation on the standardized ileal digestibility of amino acids and apparent metabolizable energy in peanut meal fed to broiler chickens[J]. *Fermentation*, 2023, 9(4):346.

(4) The application of FPNM in the diets of broilers, particularly its effects on growth performance and other aspects. (**Chapter VII**)

Chapter IV

Screening and characterization of *Bacillus* spp. based on the ability to produce multi-enzymes

Based on the characteristics of peanut meal, it is necessary to screen the bacteria suitable for fermentation. One selected strain should possess the potential to extracellularly secrete protease, cellulase, and phytase enzymes, enabling the biotransformation of peanut meal. Furthermore, it is essential for the bacterium to be safe for poultry.

This chapter is based on the following publication:

Chong Li, Shuzhen Li, Guoqi Dang, Rui Jia, Si Chen, Xuejuan Deng, Guohua Liu, Yves Beckers and Huiyi Cai

Screening and characterization of *Bacillus velezensis* LB-Y-1 toward selection as a potential probiotic for poultry with multi-enzyme production property

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Author Contributions: C.L., S.L. and G.D., Conceptualization; C.L. and S.L., Methodology; C.L., G.D. and R.J., investigation, software, data curation and writing-original draft preparation; S.C. and X.D., validation and resources; G.L. and Y.B., writing review, editing and supervision; H.C., project administration and funding acquisition.

Chapter IV. Screening and characterization of *Bacillus* spp. based on the ability to produce multi-enzymes

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Key words: *Bacillus velezensis*, multi-enzyme, broiler chickens, tibia mineralization, digestive enzymes, intestinal microbiota

1. Abstract

Bacillus spp. have gained increasing recognition as an option to use as antimicrobial growth promoters, which are characterized by producing various enzymes and antimicrobial compounds. The present study was undertaken to screen and evaluate a *Bacillus* strain with the multi-enzyme production property for poultry production. LB-Y-1, screened from the intestines of healthy animals, was revealed to be a *Bacillus velezensis* by the morphological, biochemical, and molecular characterization. The strain was screened out by a specific screening program, possessed excellent multi-enzyme production potential, including protease, cellulase and phytase. Moreover, the strain also exhibited amylolytic and lipolytic activity *in vitro*. The dietary LB-Y-1 supplementation improved growth performance and tibia mineralization in chicken broilers, and increased serum albumin and serum total protein at 21 d of age ($p < 0.05$). Besides, LB-Y-1 enhanced the activity of serum alkaline phosphatase and digestive enzyme in broilers at 21 and 42 d of age ($p < 0.05$). Analysis of intestinal microbiota showed that a higher community richness (Chao1 index) and diversity (Shannon index) in the LB-Y-1 supplemented compared with the CON group. PCoA analysis showed that the community composition and structure were distinctly different between the CON and LB-Y-1 group. The beneficial genera such as *Parasutterella* and *Rikenellaceae* were abundant, while the opportunistic pathogen such as *Escherichia-Shigella* were reduced in the LB-Y-1 supplemented group ($p < 0.05$). Collectively, LB-Y-1 can be considered as a potential strain for further utilization in direct-fed microbial or starter culture for fermentation.

2. Introduction

"Beneficial" "friendly" and "healthy" are commonly used to describe probiotics. Previous studies have proved that probiotics may have a fundamental impact on the maturation of animal immune phenotype, and the metabolisms of different functional microorganisms would play a crucial role in the maintenance of health (Gao et al., 2017; Ma et al., 2018). Therefore, it is imperative to screen functional microorganisms with excellent characteristics and explore their roles in different periods of life (Kundu et al., 2017; Francino, 2018; Tun et al., 2018). One of functional properties of microorganism is the ability to secrete multi-enzyme by exocytosis, and these enzymes are able to further degrade certain nutrient substances that are not well digested by endogenous enzymes produced in the animal's digestive system (Lee et al., 2020). For example, the proteases can convert insoluble storage protein into soluble peptides and amino acids (Kesari and Rangan, 2011). The cellulases such as cellobiohydrolases, β-glucosidases, endoglucanases can degrade the fiber components in feed (Stålbrand et al., 1998; Ye et al., 2017). Microbial phytases can hydrolyze the phytate complex and release the nutrients for use by the broilers (Babatunde et al., 2021; Li et al., 2022a).

One of main objectives in the poultry industry is to maximize the productivity. However, several major issues confront the poultry meat production today, such

as the imbalance in protein resources, and viral or bacterial infectious diseases. In parallel, the global trend for reducing antibiotic growth promoters in the animal production has gathered momentum (Pourabedin et al., 2015). It is particularly important to explore the nutritional interventions for the prevention of these pathological processes, and the supplementation of exogenous amylase, protease or other enzymes for broiler diets could be a strategic approach (Chang'a et al., 2019; Córdova-Noboa et al., 2021). Studies have shown that a number of *Bacillus* spp. can be added to feed, which has the effect of improving animal growth performance, regulating the intestinal micro-ecology and promoting the utilization of nutrients (Lee et al., 2008; Li et al., 2022a).

Researchers have confirmed that the *Bacillus velezensis* (*B. velezensis*) has the characteristics of producing a variety of enzymes like protease, cellulase, amylase and glucanase, as well as secreting antibacterial substances to inhibit the growth of pathogenic microorganisms (Meng and Hao, 2017; Li et al., 2018b). However, a comprehensive evaluation of the effect of *B. velezensis* on poultry is lacking. In the current study, *B. velezensis* LB-Y-1 with the ability to produce multi-enzyme including protease, cellulase and phytase was screened and characterized from the intestinal tract of different healthy animals. Ultimately, the strain was comprehensively evaluated for its safety and efficacy in growing broilers.

3. Materials and methods

3.1 Sample collection and isolation of *Bacillus* spp.

The strains were isolated from the digestive tracts of healthy and free-ranging animals (the rumen of cattles, and the cecum of chickens, pigs and rabbits) without any additives during the rearing period (such as antibiotics or probiotics). All strains were collected in accordance with the Bioconvention and the Nagoya protocol, 2014. *Bacillus* spp. was isolated according to the method described as previously, with minor modification (Susanti et al., 2021; Liu et al., 2022). Heat the intestinal contents at 95 ℃ for 5 minutes in order to separate out the *Bacillus* spores, followed by a 10-fold series dilution with sterile normal saline to ensure recovery of individual colonies on agar plates. Isolation was performed using agar spot test on Luria-Bertani (LB)-casein medium (Hope Bio-Technology Co., Ltd., Qingdao, China; casein, 0.4%). Based on the colony morphology and casein-soluble region of *Bacillus* spp., 191 colonies were purified and selected. The strains were grown in LB broth (Hope Bio-Technology Co., Ltd., Qingdao, China) and stored in LB broth with 20 % glycerol at -80 ℃.

3.2 Protease production capacity (primary screening)

The protocol was based on the previous method with minor modification (Liu et al., 2022). Briefly, the selected strains were cultured at 37 ℃ for 24 h and the inoculum was added at 10% (v/v) rate to the fermentation broth containing 20 g peanut meal and 20 ml distilled water in a flask. The cultures were incubated anaerobically at 37 ℃ for 72 h. The fermentation product (10 g) was added to a 250 mL Erlenmeyer flask containing 90 mL of distilled water and incubated in

40 ℃ water bath for 1 h (stirred every 15 min). The crude enzyme solution was obtained after filtration through filter paper Whatman No.1 (Whatman International Ltd, Maidstone, UK). Folin-Ciocalteu method was used to determine the protease activity (Ramkumar et al., 2018). All tests were repeated four times.

3.3 Cellulase production capacity (secondary screening)

The cellulase-production strains were screened by the carboxymethylcellulose (CMC) plate assay method (Shaikh et al., 2013; Mohammad et al., 2017). In brief, the plates were incubated at 37 ℃ for 5 days to produce enough cellulase, then the plates were incubated in Congo red solution (1% w/v) for 15 min at 37℃, followed by flooding with 1 M NaCl for 15 min. The strains with cellulose degrading ability showed a clear zone. The ratio of clear zone diameter to colony diameter (mm/mm) was considered as degradation efficiency. All tests were repeated four times.

3.4 Phytase production capacity (tertiary screening)

The phytase-production strains were screened following the method explained by (Demirkan et al., 2014). Firstly, the freshly cultured strains in LB broth (OD_{600}=0.3) were inoculated into enzyme production broth (Dextrose 0.5%, yeast extract 0.5%, peptone 1.0%, sodium phytate 0.1%, $CaCl_2$ 0.1%, $MgSO_4$ 0.1% (w/v, pH 7.0)) at 1% rate, and then incubated at 37 ℃, 150 rpm for 24, 32, 48, 56, 72 and 80 h in a shaking incubator. At the end of each period, the culture supernatant was collected for determination of phytase activity using the method explained by (Choi et al., 2001). All tests were repeated four times, and selected the strains with high phytase activity for further analysis.

3.5 Morphological, biochemical, and molecular characterization of LB-Y-1

The characterizations of LB-Y-1 were done using the method explained by Zhang et al., (2021a) with minor modifications. The characteristic colonies were gram stained, and bacteria were examined for morphology under a light microscope (Olympus CX40, Olympus Optical Co. Ltd., Hamburg, Germany) and a scanning electron microscopic (SEM, Inspect F50, FEI Company, Hillsboro, OR, USA) (Bajpai et al., 2016b). The growth curve assay of LB-Y-1 was performed according to the method described previously (Fitzgerald et al., 2020). Supplementary File 1 showed the molecular biology identification procedure for LB-Y-1. A bootstrap phylogenetic tree was constructed by the neighbor-joining method, using MEGA 7 software (www.megasoftware.net). Furthermore, biochemical characteristics of LB-Y-1 were performed using the API 50 CHB system (BioMerieux Vitek, France) according to the instructions from the manufacturer.

In order to further examine the potential of LB-Y-1 to produce multi-enzymes, the activity of amylase and lipase in the LB-Y-1 were determined using the solid agar plate method (Zhang et al., 2021a). Briefly, after cultured in LB broth at 37 ℃ overnight, the strain was stab-inoculated on the surface of LB agar plate containing 1.5% soluble starch or 1% triglyceride tributyrate (four replicates were made of each assay). The plates were subsequently incubated at 37℃ for 24 h.

The amylase and lipase activities were determined based on the diameter of the transparent halos.

3.6 Safety evaluation of LB-Y-1 (*in vitro*)

3.6.1 Haemolytic activity assay

The haemolytic activity of LB-Y-1 was determined using the method explained by Maragkoudakis et al., (2006), with minor modifications. Briefly, LB-Y-1 was cultured in LB broth overnight, and then inoculated in pre-made blood agar (Beijing Land Bridge Technology Co., Ltd., Beijing, China) containing 5% (v/v) sheep blood. The presence of a hemolysis zone was observed following incubation at 37 ℃ for 24 h.

3.6.2 Antibiotic susceptibility

Antibiotic susceptibility of LB-Y-1 was determined by the agar diffusion method of CLSI, (2015), with minor modifications. Briefly, LB-Y-1 was cultured in LB broth at 37 ℃ for 12 h, and then preparing a suspension in accordance with two McFarland's scales (10^8 CFU/mL) (Reuben et al., 2019). A total of 100 μL suspension were spread onto MRS agar plates, in which the antibiotic discs were placed. The commercial antibiotic discs (HANGWEI, Hangzhou, China) contains Cefperazone (75 μg), Ceftriaxone (30 μg), Ceftazidime (30 μg), Cefuroxime (30 μg), Cefradine (30 μg), Cefazolin (30 μg), Cefalexin (30 μg), Minocyline (30 μg), Doxycycline (30 μg), Tetracycline (30 μg), Ciprofloxacin (5 μg), Clindamycin (2 μg), Erythromycin (15 μg), Neomycin (30 μg), Kanamycin (30 μg), Gentamicin (10 μg), Amikacin (30 μg), Vancomycin (30 μg), Piperacillin (100 μg), Ampicillin (100 μg), Oxacillin (1 μg), Penicillin (10 μg), Chloramphenicol (30 μg), Furazolidone (300 μg). Plates were incubated for 24 h at 37 ℃ and the diameters of the clear zones were measured and classified as sensitive (S), intermediate (I), and resistance (R) according to the guidelines for CLSI.

3.7 *In vivo* testing

3.7.1 Strain preparation

The strain *B. velezensis* LB-Y-1 was emulsified into microcapsules (prepared by Challenge Biotechnology Co., LTD (Beijing, China, viable count $\geq 5.0\times10^{10}$ CFU/g). Following a conservative strategy, the amount of LB-Y-1 in feed was examined daily throughout the experiment to ensure cell viability (Nikoskelainen et al., 2003).

3.7.2 Experimental design and bird management

The research was conducted at Nankou pilot base of the Chinese Academy of Agricultural Sciences (CAAS). The animal experiments were performed under the National Institute of Animal Health approved protocol and the ARRIVE guidelines were followed for reporting results (Kilkenny et al., 2012).

A total of 120 one-day-old male Arbor Acres (AA) broiler chickens (body weight, 40.2±0.4 g) were randomly allocated into 2 treatment groups with 6 replicates of 10 birds each replicate. The control (CON) group were fed a

corn-soybean-based diet, which met the nutritional requirements of broilers (Supplementary Table S1) in pellet form. LB-Y-1 homogenate was added at 100mg/kg to the basal diets (BV group), and the final concentration was 3.5×10^9 CFU/kg. The mixing and pelleting were operated by a single trained person and fresh diets were produced every 3 days to ensure uniformity of additives. The experiment lasted for 42 days in two feeding phases, starter (1-21 d) and grower (22-42 d), and all the broilers were housed in the same environmentally controlled facility (cleaning cage equipped with the fiberglass feeders and plastic net floor). All broilers were allowed ad libitum feed and water, and given the same photoperiod (16 h light: 8 h dark), relative humidity (1-7 d, 60%-70%; 8-42 d, 50%-60%) and room temperature (1-7 d, 33±2 ℃; 8-16 d decreased stepwise to 24 ℃; 17-42 d 24 ℃).The excreta was cleared daily. Broilers were subjected a routine vaccination program, and their health was monitored daily.

3.7.3 Sampling

Body weight (BW) were measured at 21 and 42 d of age and record the feed intake, average daily feed intake (ADFI), average daily gain (ADG) and the feed intake/weight gain (F/G) ratio were calculated for the different phases. At 21 and 42 d of age, one bird close to the average body weight from each replicate was selected after 12 hours fasting. Blood samples (2.5 mL) were collected from the wing vein in the EDTA-containing (5 mL). Serum was harvested from nonanticoagulated whole blood by centrifuging at 3,000×*g* for 10 min, and stored at -20 ℃ until analyzed. The slaughter performance and immune organ indexes were measured according to the production performance noun terms and metric statistics method of poultry (NY/T823-2004)(Liu et al., 2021; Gao et al., 2022c). One side of the tibia bone were removed for mineralization analysis. The jejunum digesta samples were collected from the middle of the jejunum and stored at -20 ℃ until further analysis. Liver, spleen and intestinal tissues (jejunum and ileum) were collected and fixed in 10% buffered formaldehyde (pH 7.4) for histological analysis. At 42 d of age, the ileal contents of 6 broilers were collected and snap-frozen in liquid nitrogen, followed by storage at -80 ℃ for DNA extraction.

3.7.4 Hematological and serum biochemical indexes analysis

Using an auto hematology analyzer (Sysme XT-1800i, China) to detect red blood cell count (RBC), white blood cell count (WBC), lymphocytes (LYM) and hemoglobin concentration (HGB). The alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and P level were measured using an automatic blood biochemical analyzer (Olympus AU640, Japan). The total protein (TP) and albumin levels of serum were measured using commercial assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) by colorimetric method. Because the TP in serum mainly consists of albumin and globulin, the globulin content was obtained by subtracting the albumin value from that of the TP (Salem et al., 2018).

3.7.5 Intestinal digestive enzyme and tibia bone mineralization analysis

Amylase, trypsin and lipase activities of the digesta samples were measured by using the commercial assay kits (C016, A080 and A054, Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Tibia bone mineralization analysis involved measuring ash of tibia bone and P and Ca concentration in the bone ash, according to the method reported by (Li et al., 2022a).

3.7.6 Histological analysis

Fixed intestine samples were dehydrated in various concentrations of ethyl alcohol (75%, 85%, 95%, and 100%), cleared in xylene, and embedded in paraffin. Five micrometer-thick sections were prepared using a microtome, then the paraffin-embedded sections were deparaffinized, dehydrated and stained by hematoxylin-eosin (H&E). Visualization was performed under a light microscope (Olympus CX40, Olympus Optical Co. Ltd., Hamburg, Germany). For the jejunum and ileum sections, epithelial thickness, villus length and crypt depth were measured at least 10 well-oriented villi, and the villus length/crypt depth ratio (V/C) ratio was calculated.

3.7.7 Microbial analysis

Total genomic DNA was extracted from the intestinal contents using commercially kit (Qiagen, Hilden, Germany), and the quality of DNA was checked by agarose gel electrophoresis. DNA concentration and purity were investigated using a NanoDrop2000 spectrophotometer (Thermo Fisher Scientific, Waltham, USA). The V3-V4 hypervariable regions from 16S rRNA gene was amplified with universal primers 338F (ACTCCTACGGGAGGCAGCAG) and 806R (GGACTACHVGGGTWTCTAAT). The amplicon was PCR purified and quantified as previously described (Takeshita et al., 2016). The library preparation and sequencing were carried out by Majorbio Biotech Co., Ltd (Shanghai, China) using the Illumina MiSeq platform (Illumina, Madison, USA). The original data were subjected to quality control and species annotation processes to obtain effective Tags. Sequences were clustered into operational taxonomic units (OTUs) with 97% consistency, diversity and taxonomy was obtained by using QIIME software (version 1.9.1). Data were analyzed using the online platform of Majorbio Technology Co., Ltd (https://cloud.majorbio.com/). The alpha-diversity analysis was based on the normalized data including Chao1 index, Shannon index, Coverage index and numbers of OUTs. The beta-diversity was presented using principal coordinate analysis (PCoA) (Lozupone and Knight, 2005). The relative abundance of the relative abundance at the phylum and genus levels were analyzed by the Student's t-test.

3.8 Statistical analysis

All experimental data were tested for normality by using the Shapiro-Wilk normality test and for homogeneity of variances by using Levene's test of SPSS19.0 software package for Windows (SPSS Inc., Chicago, IL, USA). Afterward, the data were analyzed by student's t-test or one-factor analysis of

variance (ANOVA) where appropriate. $p < 0.05$ showed a statistically significant. For the indexes expressed as means with standard error of mean (SEM).

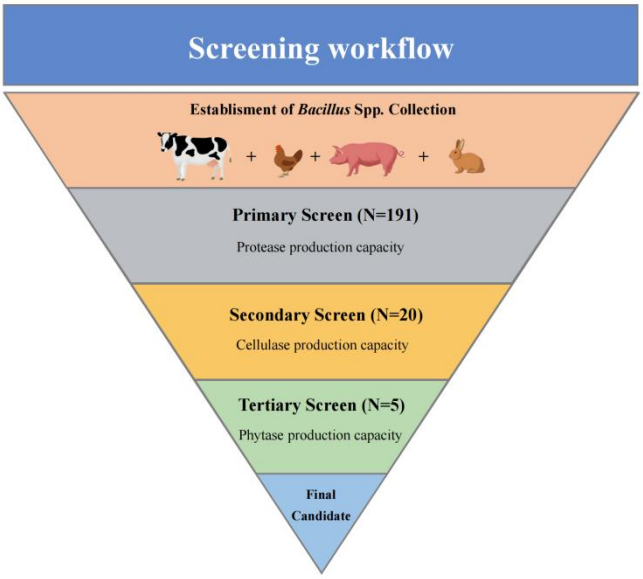


Figure 5. Workflow of the screening. The process was divided into three parts and performed to narrow down a total of 191 *Bacillus* spp. isolates to one strain with the ability to produce multi-enzyme

4. Results

A total of 191 potential *Bacillus* spp. were isolated. The screening process was composed of three steps, which was enabled to detcct *Bacillus* spp. with the best multi-enzyme production properties from all candidates, the workflow is given in Figure 5.

4.1 Protease production capacity (primary screening)

In the primary screening, potential strains with strong protease production capacity were selected, and the protease activity depended on source of origin and the specific strain. The best 20 isolates with superior protease production potential that are depicted in Figure 6A were further screened.

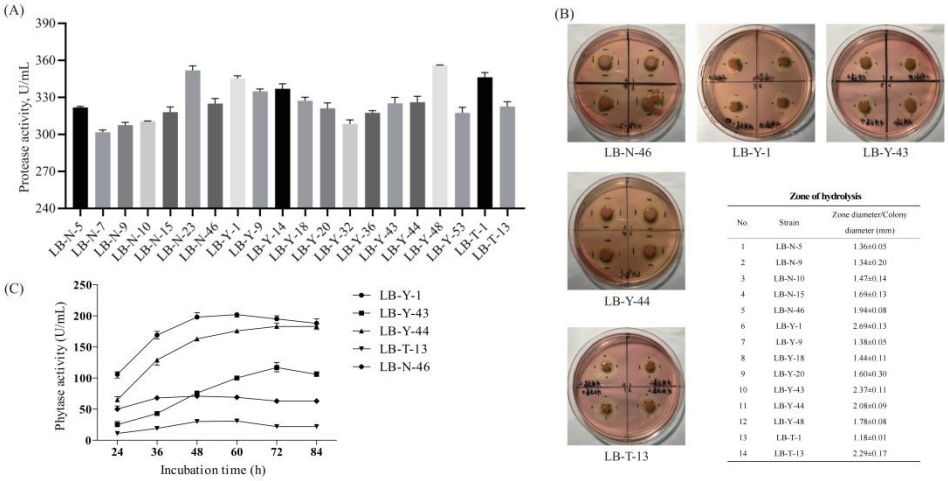
4.2 Cellulase production capacity (secondary screening)

After the primary screening, the selected *Bacillus* spp. were tested for cellulase production capacity. A total of 14 strains were screened through the CMC plate assay method (Figure 6B), among which, 5 strains were found to have the largest degradation zone. Therefore, these strains (LB-N-46, LB-Y-1, LB-Y-43, LB-Y-44 and LB-T-13) were the best candidates for cellulase production capacity, and were further analyzed.

4.3 Phytase production capacity (tertiary screening)

The capacity of phytase production of the candidates is depicted in Figure 6C. Strains LB-Y-43, LB-T-13 and LB-N-46 exhibited low phytase activity during the

culture, while LB-Y-1 and LB-Y-44 showed a higher activity, and LB-Y-1 reached the highest enzyme activity at earlier time point (48h). Through the screening and assessment process, LB-Y-1 was selected as a potential strain for subsequent experiments.



Zone of hydrolysis

No.	Strain	Zone diameter/Colony diameter (mm)
1	LB-N-5	1.36±0.05
2	LB-N-9	1.34±0.20
3	LB-N-10	1.47±0.14
4	LB-N-15	1.69±0.13
5	LB-N-46	1.94±0.08
6	LB-Y-1	2.69±0.13
7	LB-Y-9	1.38±0.05
8	LB-Y-18	1.44±0.11
9	LB-Y-20	1.60±0.30
10	LB-Y-43	2.37±0.11
11	LB-Y-44	2.08±0.09
12	LB-Y-48	1.78±0.08
13	LB-T-1	1.18±0.01
14	LB-T-13	2.29±0.17

Figure 6. The screening process of *Bacillus* spp. (A) Protease production capacity (Primary Screen). (B) Cellulase production capacity (Secondary Screen). (C) Phytase production capacity (tertiary Screen). Values are presented as Mean ± SEM

4.4 Morphological, biochemical, and molecular characterization of LB-Y-1

The colony morphologies of LB-Y-1 was wet, opaque, wrinkled and irregular on the edges on the surface of LB agar, which was confirmed as gram-positive cocci (elongate oval) by microscopic evaluation (Figure 7A and 7B). The cells appear frequently in pairs or short chains (Figure 7C). These features suggested that it was related to *Bacillus* spp. The proliferation curves appeared as typically sigmoidal shape, consisting of latency phase (0-6 h), logarithmic phase (6-20 h) and plateau phase (20 h later) (Figure 7D). Furthermore, 16S rRNA gene sequencing and phylogenetic analysis found the LB-Y-1 share 100% similarity with the sequences of *B. velezensis* BCRC-17467^T (Figure 7E). Biochemical analysis revealed that, the main physiological and biochemical characteristics of the LB-Y-1 were similar to *B. velezensis* WLYS23 (Supplementary Table S2). Collectively, LB-Y-1 was identified as *B. velezensis* and deposited it in the China General Microbiological Culture Collection Center (CGMCC, Beijing, China) with accession number 21344. Furthermore, the LB-Y-1 formed hydrolytic circles on the media in the assays for the degradation of starch and triglyceride (Figure 7F and 7G), indicating that the strain also has the capacity to produce amylase and lipase.

4.5 Safety evaluation of LB-Y-1 (*in vitro*)

The hemolytic activity of LB-Y-1 was judged by observing the hemolytic rings on blood agar plates after an 24 h incubation. The strain was not involved in the

lysis of erythrocytes (results not shown). Supplementary Table S3 showed the antibiotic susceptibility profile of the LB-Y-1. It was susceptible to 24 routinely used antimicrobials, indicating that the strain is safe and can be used as a probiotic.

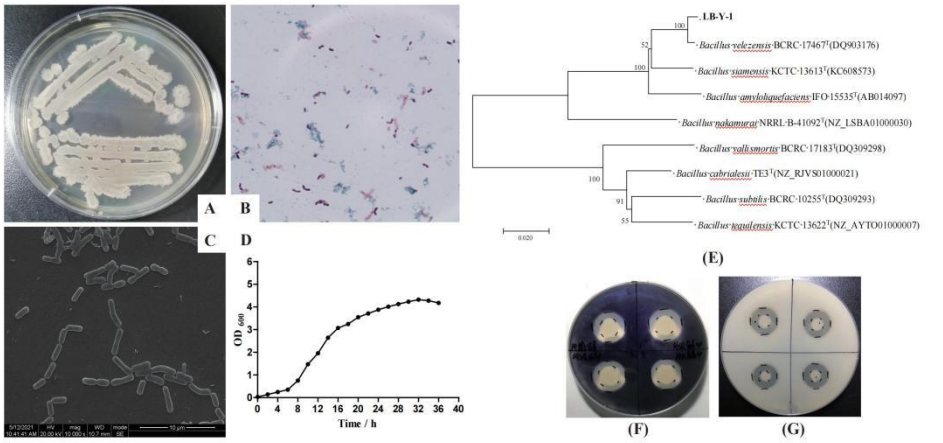


Figure 7. Morphological, biochemical, and molecular characteristics of LB-Y-1. (A) Colony morphology. (B) Gram stain showing Gram-positive rod. (C) SEM image of LB-Y-1 showing a small rods morphology, frequently in pairs or short chains (×1,000). (D) The growth curve of LB-Y-1. (E) The phylogenetic tree analysis of LB-Y-1. (F) Starch-degradation ability. (G) Triglyceride-degradation ability

4.6 *In Vivo* testing

4.6.1 Growth performance

The average mortality rate was 0.5% during the experiment (data not presented) with no significant difference between the groups. Growth performance is depicted in Figure 8. Compared with CON group, LB-Y-1 significantly increased the BW of broilers at 21 and 42 d of age, and increased the ADG during the starter, grower and overall periods ($p < 0.05$). The F/G ratio for the BV group was lower than the CON group during the whole period ($p < 0.05$). No significant differences were found in the ADFI between the groups.

4.6.2 Slaughter performance and immune organ indexes

The characteristics of slaughter performance was presented in Table 5, no significant difference was detected between the groups. In addition, supplementation with LB-Y-1 had no effect on immune organ indexes at 21 and 42 d of age compared to the CON group (Table 6).

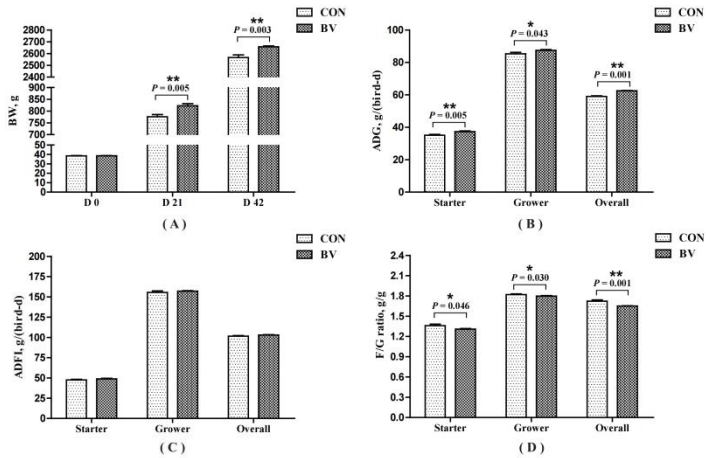


Figure 8. Effect of LB-Y-1 on growth performance of broilers (n = 6). CON = control group, broilers were fed a corn-soybean basal diet, BV = *B. velezensis* LB-Y-1 group, broilers were fed a basal diet containing 3.5×10^9 CFU/kg LB-Y-1. BW, body weight; ADG, average daily gain; ADFI, average daily feed intake; F/G ratio = feed intake (g)/weight gain (g); Values are presented as Mean ± SEM, the values having superscript (*) were significantly different, *$0.01 < p < 0.05$; **$p \leq 0.01$.

Table 5. Effect of LB-Y-1 on slaughter performance of broilers at day 42 (n=6)

Items	CON	BV	SEM	p-value
Dressing percentage, %	88.73	90.03	0.363	0.556
Half-eviscerated yield, %	84.92	84.84	0.219	0.975
Eviscerated yield, %	77.59	76.72	0.363	0.596
Breast muscle ratio, %	13.03	13.10	0.303	0.383
Thigh muscle ratio, %	11.03	11.22	0.580	0.856
Abdominal fat ratio, %	2.41	2.11	0.038	0.970

CON=control group, broiler chickens were fed a corn-soybean basal diet, BV=*B. velezensis* group, broiler chickens were fed a basal diet containing 3.5×10^9 CFU/kg LB-Y-1. SEM=standard error of means.

4.6.3 Hematological and serum biochemical indexes analysis

As shown in Table 7, LB-Y-1 treatment significantly increased the levels of ALP (p=0.011, 21 d of age and p=0.007 42 d of age), TP (p=0.001, 21 d of age),

Albumin (p=0.002, 21 d of age), A/G ratio (p=0.031, 21 d of age) and P (p=0.001, 42 d of age) compared to the CON group.

Table 6. Effect of LB-Y-1 on immune organ indexes of broilers (n=6)

Items	Dietary treatment		SEM	p-value
	CON	BV		
Day 21				
Spleen index, mg/g	0.91	0.89	0.029	0.896
Thymus index, mg/g	2.86	2.84	0.021	0.709
Bursa of fabricius index, mg/g	1.71	1.74	0.020	0.646
Day 42				
Spleen index, mg/g	1.20	1.21	0.041	0.649
Thymus index, mg/g	2.15	2.16	0.015	0.789
Bursa of fabricius index, mg/g	1.13	1.19	0.016	0.146

CON=control group, broiler chickens were fed a corn-soybean basal diet, BV=*B. velezensis* group, broiler chickens were fed a basal diet containing 3.5×10^9 CFU/kg LB-Y-1. Spleen index, thymus index and bursa of fabricius index were calculated as follows: (1) Spleen index (mg/g)=spleen weight (mg)/body weight (mg), (2) Thymus index (mg/g)=thymus weight (mg)/body weight (g), (3) Bursa of fabricius index (mg/g)=bursa of fabricius (mg)/body weight (g). SEM=standard error of means.

Table 7. Effect of LB-Y-1 on hematological and serum biochemical indexes of broilers (n=6)

Items	Dietary treatment		SEM	p-value
	CON	BV		
Day 21				
RBC, $\times10^{12}$/L	2.59	2.64	0.083	0.197
HGB, g/L	101.24	102.83	3.126	0.766
WBC, $\times10^9$/L	141.57	139.61	1.593	0.097
LYM, $\times10^9$/L	55.01	55.72	1.020	0.162
ALT, U/L	2.31	2.19	0.017	0.356
AST, U/L	243.67	241.05	1.207	0.609
ALP, U/L	2872.67	2960.17*	18.851	0.011
Phosphorus, mmol/L	1.50	1.54	0.011	0.065

TP, g/L	3.09	3.26**	0.021	0.001
Albumin, g/L	1.34	1.48**	0.018	0.002
Globulin, g/L	1.75	1.78	0.010	0.297
A/G ratio	0.77	0.83*	0.010	0.031
Day 42				
RBC, $\times10^{12}$/L	2.47	2.50	0.080	0.497
HGB, g/L	100.53	99.67	3.372	0.441
WBC, $\times10^{9}$/L	142.52	139.35	1.354	0.085
LYM, $\times10^{9}$/L	57.38	59.20	0.946	0.246
ALT, U/L	3.25	3.17	0.020	0.082
AST, U/L	239.85	232.43	1.343	0.185
ALP, U/L	2789.83	2880.67**	18.831	0.007
Phosphorus, mmol/L	1.45	1.53*	0.016	0.001
TP, g/L	3.15	3.22	0.026	0.159
Albumin, g/L	1.39	1.43	0.016	0.095
Globulin, g/L	1.76	1.79	0.013	0.446
A/G ratio	0.79	0.80	0.007	0.274

CON=control group, broiler chickens were fed a corn-soybean basal diet, BV=*B. velezensis* group, broiler chickens were fed a basal diet containing 3.5×10^{9} CFU/kg LB-Y-1. RBC, red blood cell count. HGB, hemoglobin concentration. WBC, white blood cell count. Lym, lymphocytes. ALT, alanine aminotransferase. AST, aspartate aminotransferase. TP, total protein. A/G ratio=albumin/globulin. SEM=standard error of means. * (p<0.05) and ** (p<0.01) indicate a significant difference within row.

4.6.4 Intestinal digestive enzyme

The effect of LB-Y-1 on intestinal digestive enzyme of broilers is depicted in Table 8. Broilers with LB-Y-1 had higher amylase and trypsin activity at 21 d of age and higher amylase activity at 42 d of age compared to the CON group (p < 0.05).

Table 8. Effect of LB-Y-1 on digestive enzyme activity of broilers (n=6)

Items	Dietary treatment		SEM	p-value
	CON	BV		
Day 21				
Amylase (U/mg protein)	174.17	205.08**	5.709	0.001
Trypsin (U/mg protein)	4374.04	4621.04**	48.410	0.003
Lipase (U/g protein)	26.51	26.70	0.174	0.626

Day 42				
Amylase (U/mg protein)	123.08	144.75*	4.861	0.017
Trypsin (U/mg protein)	4758.05	4897.69	53.050	0.202
Lipase (U/g protein)	23.28	22.47	0.355	0.271

CON=control group, broiler chickens were fed a corn-soybean basal diet, BV=*B. velezensis* group, broiler chickens were fed a basal diet containing 3.5×10^9 CFU/kg LB-Y-1. SEM=standard error of means. * ($p<0.05$) and ** ($p<0.01$) indicate a significant difference within row.

4.6.5 Tibia bone mineralization

The effect of LB-Y-1 on tibia bone mineralization of broilers is depicted in Figure 9. Supplementation with LB-Y-1 significantly increased tibia bone ash at 42 d of age, along with tibia ash P concentration compared to control broilers ($p < 0.05$). Although not significant, tibia ash Ca concentration was also elevated in the BV group relative to broilers of CON group.

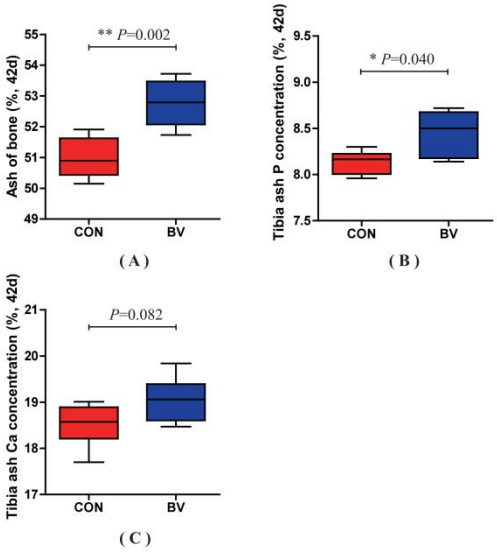


Figure 9. Effect of LB-Y-1 on tibia bone mineralization of broilers ($n = 6$). (A) Bone ash content at 42 days of age. (B) Tibia ash P concentration at 42 days of age. (C) Tibia ash Ca concentration at 42 days of age. Values are presented as Mean ± SEM, the values having superscript (*) were significantly different, *$p < 0.05$; **$p < 0.01$.

4.6.6 Histological analysis

Table 9 showed the effect of LB-Y-1 on intestinal histomorphology of broilers. LB-Y-1 significantly decreased the crypt depth of jejunum, and increased the ratio of villus height to crypt depth at 21 d of age ($p < 0.05$). There were no significant differences in histomorphometric features of the jejunum and ileum between the

CON and BV groups at 42 d of age. Furthermore, LB-Y-1 had no significant effect on epithelial thickness of jejunum and ileum at 21 and 42 d of age.

Table 9. Effect of LB-Y-1 on histomorphology of the small intestinal sections in broilers (*n*=6)

Items	Intestine	Treatment		SEM	*p*-value
		CON	BV		
Day 21					
Jejunum	Villus length, μm	1259.88	1164.63	28.512	0.095
	Crypt depth, μm	183.65**	153.32	5.746	0.002
	V/C ratio	6.86	7.62**	0.174	0.021
	Epithelial thickness, μm	218.15	227.98	6.046	0.443
Ileum	Villus length, μm	805.98	773.19	12.126	0.188
	Crypt depth, μm	197.87	186.35	4.258	0.187
	V/C ratio	4.09	4.15	0.087	0.747
	Epithelial thickness, μm	224.77	214.89	5.246	0.371
Day 42					
Jejunum	Villus length, μm	1295.59	1210.03	27.473	0.123
	Crypt depth, μm	213.20	210.51	4.932	0.799
	V/C ratio	6.09	5.77	0.126	0.219
	Epithelial thickness, μm	279.57	255.17	7.758	0.119
Ileum	Villus length, μm	831.92	818.94	6.493	0.341
	Crypt depth, μm	209.55	192.72	4.900	0.085
	V/C ratio	3.99	4.27	0.095	0.153
	Epithelial thickness, μm	346.03	325.97	10.154	0.347

CON=control group, broiler chickens were fed a corn-soybean basal diet, BV=*B. velezensis* group, broiler chickens were fed a basal diet containing 3.5×10^9 CFU/kg LB-Y-1. V/C ratio=Villus length (μm)/Crypt depth (μm). SEM=standard error of means. * ($p<0.05$) and ** ($p<0.01$) indicate a significant difference within row.

4.6.7 Microbial analysis

To understand whether LB-Y-1 could modulate gut microbiota community, we investigated the change of the ileal microbiota diversity. A community richness (Chao1 index) and diversity (Shannon index) analysis demonstrated that broilers supplemented with the LB-Y-1 had significantly higher microbial relative abundance and potential diversity than those of CON group ($p<0.05$) (Figures

10A and 10B). The coverage index was greater than 0.998 in each group (Figure 10C), indicating an adequate depth of sequencing.. Furthermore, a higher OTU richness was observed in the BV group (Figure 10D). The *β*-diversity analysis indicated that samples in CON and BV groups had different community composition and structure, suggesting a significant segregation of microbiota between the groups (Figure 10E). Firmicutes was the predominant phyla (Figure 10F), and *Lactobacillus* and *Enterococcus* were the main dominant genera (Figure 10G). The LEfSe analysis (LDA>2) revealed the significant differences in microbiota structure between the CON group and the BV group (Figure 11A). Student's t-test showed that BV group had a lower abundance of Proteobacteria, and higher abundance of Cyanobacteria and Bacteroidota at phylum level ($p < 0.05$, Figure 11B); as well as BV group had lower abundance of *Lactobacillus* and *Escherichia-Shigella*, and higher abundance of *Parasutterella* and *Rikenellacea*e at genus level ($p < 0.05$, Figure 11C).

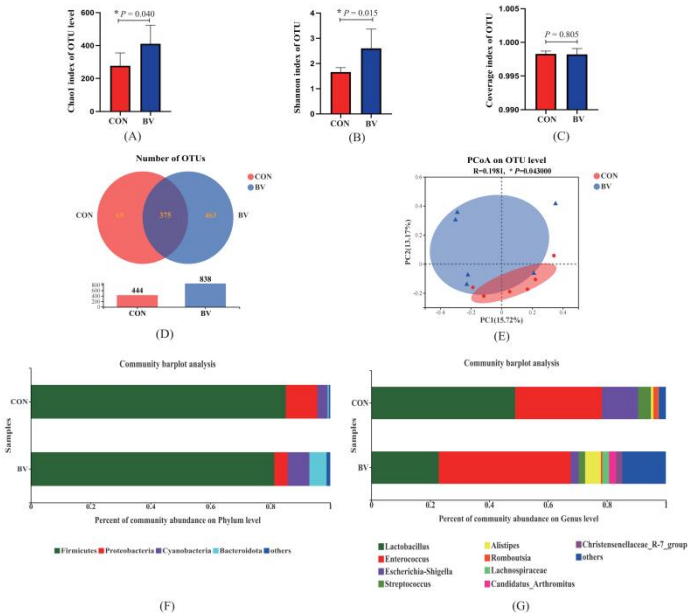


Figure 10. Effect of LB-Y-1 on the ileal microbial diversity of broilers ($n = 6$). (A) Chao1 index of OUT level. (B) Shannon index of OUT level. (C) Coverage index of OUT level. (D) Number of OTUs. (E) β-diversity was estimated by the PCoA on OUT level. (F,G) The relative abundance of bacteria at the phylum and genus levels, respectively. Values are presented as Mean ± SEM, the values having superscript (*) were significantly different, *0.01 < p < 0.05; **$p \leq 0.01$.

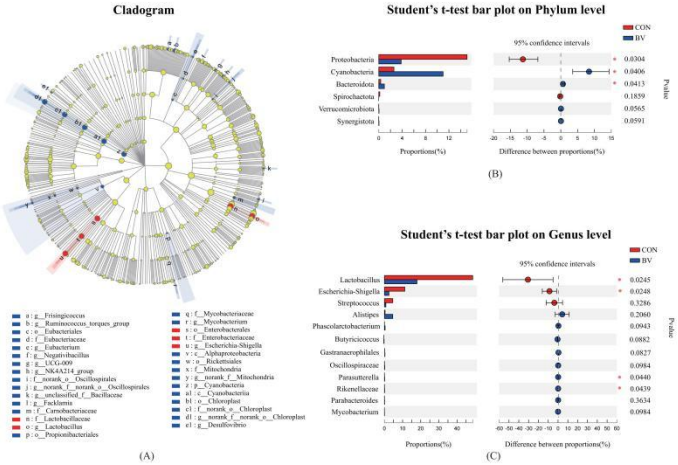


Figure 11. Effect of LB-Y-1 on the abundance of the ileal microbial community of broilers ($n = 6$). (A) Cladogram of LEfSe multilevel species difference discriminant analysis (LDA > 2), different color nodes indicate microbial communities that are significantly enriched in the corresponding groups and significantly different between groups. (B,C) Comparative analysis of the relative abundance of bacteria at the phylum and genus levels, respectively. Values are presented as Mean ± SEM, the values having superscript (*) were significantly different, *0.01 < p < 0.05; **$p \leq 0.01$.

5. Disscussion

Bacillus spp. is emerging as a promising probiotic candidate because of the following characteristics: (1) it has the ability to form as spores and has less loss during feed preparation; (2)it is easy to produce by batch (Hyronimus et al., 2000). Furthermore, *Bacillus* spp. is recognized a excellent probiotic that produces multi-enzyme, bacteriocin, and beneficial metabolites (Li et al., 2018b). Previous studies have also shown positive effects of *Bacillus* spp on nutrient utilization, intestinal integrity and growth performance of broilers (Salim et al., 2013; Rivera-Pérez et al., 2021). Due to the shortage of traditional feed and the rapid rise in the price of protein raw materials, it is particularly important to improve the utilization of protein in feed, which prompted us to screen a strain of *Bacillus* spp. that could exert a positive effect on poultry production.

It is known that incomplete digestion of proteins can lead to the multiplication of putrefactive microorganisms and the accumulation of toxic metabolites (Dallas et al., 2017). High levels of protein in excreta is not only a waste of resources, but also harmful to the environment. Based on these, the protease production efficiency of *Bacillus* spp. was used as an evaluation index in the primary

screening. Further, unlike mammals and other waterfowl, broilers have lower endogenous cellulase activity, which limits fiber digestion. The addition of exogenous fiber degrading enzymes can significantly reduce chyme viscosity and improve nutrient digestibility (Slominski et al., 2006; Zhai et al., 2020). Therefore, the cellulase production capacity was taken as the evaluation index in the second screening step. There is no doubt that microbial degradation of phytic acid is essential to prevent environmental phosphate contamination and to deal with nutritional problems in monogastric animals. Phytase is widely used to improve the efficiency of phosphate absorption in animal feed (Blüher et al., 2017). In the tertiary screening, the phytase production efficiency of *Bacillus* spp. was taken as an evaluation index. Eventually, the strain of LB-Y-1 showed the best performance. According to the morphological and molecular characteristics, we preliminary identified the target strain as *B. velezensis*. Biochemical analysis revealed that, the main physiological and biochemical characteristics of the LB-Y-1 were similar to *B. velezensis* WLYS23, which has been reported (Zhang et al., 2021a). However, unlike the WLYS23, the LB-Y-1 did not react with inulin, but it was consistent with another standard strain of *B. velezensis* CR-502^T (Ruiz-García et al., 2005). The safety evaluation of LB-Y-1 indicated that LB-Y-1 was not haemolytic and highly sensitive to common antibiotics. Furthermore, consistent with the previous report Li et al., (2018b), we also found that the LB-Y-1 had the potential to degrade starch and triglyceride components. These results indicate that LB-Y-1 has potential as a safety probiotic candidate.

Like mammals, digestive system of poultry is underdeveloped early in life and cannot secrete enough gastric acid and digestive enzymes (Zhang et al., 2022). In addition, poultry has a limited ability to digest fibre in feed compared with mammals, and high-fiber or slowly digestible protein diets will cause loss of production performance (Berrocoso et al., 2020; Zhang et al., 2021b). Fortunately, it was found that the addition of exogenous enzymes may help to solve this problem (Tavernari et al., 2008). Our study found that adding LB-Y-1 increased BW and feed conversion of broilers without affecting feed intake, which we linked to the properties of LB-Y-1 secreting multi-enzyme, and this positive effect was consistent with the results of other study (Wang et al., 2021c). The effect of intestinal digestive enzymes appear in the response of *Bacillus* spp. in two ways. On the one hand, the members of *Bacillus* spp. can secrete a variety of extracellular enzymes. On the other hand, *Bacillus* spp. can stimulate the secretion of endogenous enzymes and regulate the gut microbial flora (Bedford and Schulze, 1998). Our study indicated that the cellulase and phytase secreted by LB-Y-1 contribute to the release of nutrients in feed, the protease and amylase secreted by LB-Y-1 enhanced the intestinal digestibility, thus promoting the digestion of nutrients. It has also been shown that *Bacillus* spp. may contribute to improved morphology of intestine by increasing villus height and ratio of villus height to crypt depth (Mohamed et al., 2022). The results are consistent with our findings, LB-Y-1 improved the intestinal structure at an early stage, which may contribute to the gut health.

Study in broilers demonstrated that dietary supplementation with *Bacillus* spp.

improved mineralization of tibial (Latorre et al., 2017). In the current study ALP activity was increased when LB-Y-1 were added to the diet. ALP is a kind of enzyme involved in phosphate hydrolysis and a marker of skeletal mineralization in broilers (Tilgar et al., 2008; Escobar et al., 2022). The increased activity of ALP was accompanied by a significant increase in bone phosphorus, in addition, the level of phosphorus of broilers fed LB-Y-1 was also significantly increased, which had been proved by Li et al. with *B. amyloliquefaciens* (Li et al., 2022a). In addition, the phytase produced by LB-Y-1 can also promote the release of phosphorus from phytate complex in the feed (Viveros et al., 2002), thus improving the phosphorus utilization. These results indicated that LB-Y-1 could promote the metabolism and utilization of phosphorus.

It has previously been shown that the diversity of gut microbes contributes to microbiome homeostasis and the resistance to pathogenic microorganisms (Konstantinov et al., 2004). Besides this, the intestinal microbiota community plays a variety of roles in nutrient absorption and metabolism, immunity, as well as bone density and strength (Bielke et al., 2017; Hong et al., 2019). In this study, dietary supplementation with LB-Y-1 increased the intestinal microbial diversity (Shannon index) and the microbial community richness (Chao1 index). The PCoA showed obviously different pattern of microbial communities between the groups. The predominant bacterial phylum of ileum include Firmicutes, Cyanobacteria, Proteobacteria and Bacteroidota (Guo et al., 2021), our study observed that LB-Y-1 increased the abundance of Cyanobacteria and Bacteroidota, and decreased the abundance of Proteobacteria. Cyanobacteria is the dominant phyla in the healthy intestinal tract of mammals and poultry, which can play the role of nitrogen fixation and also contribute to the nutrient absorption in the intestines (Mandal et al., 2016; Yang et al., 2020b), so the increased abundance of Cyanobacteria indicated that LB-Y-1 could improve the structure of the flora. Bacteroidota has been extensively studied for its regulatory effect on the host, which can accelerate angiogenesis in the intestinal mucosa, enhance the host's immunity, and maintain the balance of intestinal microbiota (Cheng et al., 2022). It is well known that Proteobacteria contains a wide variety of pathogens such as *Salmonella*, *Escherichia coli*, and *Shigella*, which could exert pathogenic effects in the intestine of broilers (Mora et al., 2010). Therefore, the decrease of Proteobacteria in BV group indicated that a relatively healthy bacterial community was achieved by LB-Y-1 supplementation. Furthermore, the results of the analysis at the genus level reinforced this conclusion. *Escherichia-Shigella* with reduced relative abundance in our study is an opportunistic pathogen that is positively correlated with a variety of intestinal infections (Yang et al., 2019a). Correspondingly, we also found an increase in the abundance of some beneficial genera, such as *Parasutterella* and *Rikenellaceae. Parasutterella* is a important player in multiple gastrointestinal metabolic processes, which has been shown to have a positive role in tyrosine, cholesterol and bile acid metabolism (Ju et al., 2019). *Rikenellaceae* plays an important role in promoting the fermentation of carbohydrates and proteins *in vivo*, and can reduce the damage of intestinal

immune function and the occurrence of intestinal inflammation (Su et al., 2014; Donaldson et al., 2016). For the role in bone development, Li's report suggests that *Bacillus* spp. can modulate the gut microbiome structure to affect the biosynthesis of polyamines, which in turn can mediate the enhancement of osteoblast activity and have a positive effect on increasing bone strength (Li et al., 2022a). Overall, the results revealed that LB-Y-1 can promote the organismal development of broilers by improving the structure of intestinal microbiota.

6. Conclusion

The present study demonstrated that the newly screened and characterized *B. velezensis* LB-Y-1, which was isolated from the intestinal tract of different healthy animals, has marked multi-enzyme production property. *In vivo* broilers assay indicated that LB-Y-1 has the potential to improve broiler growth performance and tibia mineralization, the mechanism may be associated with the enhanced intestinal digestive enzyme activities, increased P retention and alterations of intestinal microbiota structure. These results are encouraging and suggesting that LB-Y-1 is a potential strain for further utilization in direct-fed microbials or probiotic starter culture.

Supplemental information

Supplemental information is available in the online version of this article.

Supplementary File 1: Molecular biological identification of LB-Y-1.

Supplementary File 2: Table S1 Analysis composition of basal diets and nutrient level (air-dry basis, %).

Supplementary File 3: Table S2 Biochemical characterization of LB-Y-1, WLYS23 and CR-502T strains.

Supplementary File 4: Table S3 Antibiotic susceptibility profile of potential probiotic B. velezensis LB-Y-1.

Chapter V

Screening and characterization of lactic acid bacteria based on acid production capacity, antibacterial activity and antioxidant capacity

In the previous chapter, we successfully screened a strain of *Bacillus* spp. LB-Y-1, which exhibited excellent potential for multi-enzyme production, including protease, cellulase, and phytase. Moving forward, we need to screen a strain of lactic acid bacteria that possesses high acid production efficiency and the potential to inhibit pathogenic bacteria. This will ensure that the fermented peanut meal has a lower pH value, enhanced antioxidant properties, and an extended storage period.

This chapter is based on the following publication:

Chong Li, Shaolong Wang, Si Chen, Xiaoying Wang, Xuejuan Deng, Guohua Liu, Wenhuan Chang, Yves Beckers and Huiyi Cai

Screening and characterization of *Pediococcus acidilactici* LC-9-1 toward selection as a potential probiotic for poultry with antibacterial and antioxidative properties

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Chapter V. Screening and characterization of lactic acid bacteria based on acid production capacity, antibacterial activity and antioxidant capacity

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Key words: *Pediococcus acidilactici*, screening, antibacterial, antioxidant; broiler chickens, intestinal microbiota

1. Abstract

Growing interest has been focused on lactic acid bacteria as alternatives to antimicrobial growth promoters, which are characterized by the production of various functional metabolites, such as antimicrobial and antioxidants compounds. The present study was undertaken to evaluate a potential probiotic from the antioxidant perspective. LC-9-1, screened from the intestines of healthy animals, was revealed to be *Pediococcus acidilactici* on the basis of its morphological, biochemical, and molecular characteristics. The strain has excellent properties, including acid-production efficiency, antibacterial performance and antioxidant activity. The safety of the strain was also evaluated. Furthermore, the experiments in broiler chickens suggested that dietary LC-9-1 supplementation improved the growth performance and decreased the abdominal fat, and enhanced the antioxidant capability and intestinal innate immunity of broilers. Analysis of intestinal microbiota showed that a higher community diversity (Shannon index) was achieved. In addition to the significantly increased relative abundances of *Pediococcus* spp., beneficial genera such as *Rothia* spp. and *Ruminococcus* spp. were abundant, while opportunistic pathogens such as *Escherichia-Shigella* spp. were significantly reduced in LC-9-1 supplemented broilers. Collectively, such in-depth characterization and the available data will guide future efforts to develop next-generation probiotics, and LC-9-1 could be considered a potential strain for further utilization in direct-fed microbial or starter culture for fermentation.

2. Introduction

Poultry meat is one of the most common animal-based food sources, and makes a significant contribution to world food security and human nutrition. However, poultry is susceptible to a variety of pathogenic microorganisms during intensive production (Neveling et al., 2017). In addition, oxidative stress is another major concern due to environmental heat stress, as well as pathological and nutritional factors, which also have a negative impact on the health and productivity of poultry (Mishra and Jha, 2019).

Lactic acid bacteria (LABs) have been one of the most extensively investigated probiotics over several years (Pasolli et al., 2020). Due to their being characterized by the production of functional metabolites, including antioxidants, organic acids and antibacterial compounds, they have been widely used in the fermentation industry and in animal production (Pan and Mei, 2010). In recent years, foodborne infection and oxidative stress have become aggravated, and LABs have been designated as an important direct-fed microbiota (DFM) or probiotic starter culture (Bajpai et al., 2016b). Therefore, the further screening LAB with specific antibacterial and antioxidant capacities is of great significance.

In the present study, several LABs were screened and characterized from the intestinal tract of different healthy animals based on the antibacterial activity against selected pathogens and antioxidant capacity. Ultimately, only one strain was comprehensively assessed to determine its safety and efficacy in growing broilers.

3. Materials and methods

3.1 Pathogenic microorganisms

The pathogenic microorganisms of *Escherichia coli* (*E. coli*) ATCC25922, *Salmonella* ATCC13076 and *Staphylococcus aureus* (*S. aureus*) ATCC6538 were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA), the *Proteus mirabilis* (*P. mirabilis*) CMCCB49005 was obtained from National Center for Medical Culture Collections (CMCC, Beijing, China). The microorganisms were cultured in nutrient broth (NB) medium, and routinely sub-cultured on NB agar (NBA) medium at 4 ℃. All the mediums were purchased from Hope Bio-Technology Co., Ltd. (Qingdao, China).

3.2 Sample collection and isolation of LAB

The strains were isolated from the digestive tracts of healthy and free-ranging animals (the rumen of cattle, and the cecum of chickens, pigs and rabbits) without any additives during the rearing period (such as antibiotics or probiotics). All strains were collected in accordance with the Bioconvention and the Nagoya protocol (Diversity). LABs were isolated using the MRS-$CaCO_3$ medium (Hope Bio-Technology Co., Ltd., Qingdao, China. $CaCO_3$, 0.4%) according to the method described as Cho et al., (2013); Pan et al., (2018) with minor modification. A total of 237 colonies were categorized on the basis of their morphological characteristics and calcium-dissolving zone. The candidate strains were stored in MRS broth (Hope Bio-Technology Co., Ltd., Qingdao, China) with 20% glycerol at −80 ℃.

3.3 Acid-producing efficiency (primary screen)

The method for evaluating LAB acidogenic efficiency was slightly modified from previous studies (Xiao et al., 2015; Melgaço et al., 2018). The selected strains were cultured at 37 ℃ for 18 h, and the inoculum was then introduced into the fermentation medium (100 g autoclaved peanut meal and 200 mL sterile brine with 40 g/L NaCl in a flask) at a rate of 4% (v/v). The cultures were incubated at 30 ℃ and 39 ℃ for 36 h under anaerobic conditions. The pH value was measured at the end of incubation using a pH-meter (FE20 pH meter, Changzhou, China). Each assay was performed in three independent tests and in triplicate.

3.4 Antibacterial activity (secondary screen)

The antibacterial activity of LC-9-1 was determined by the standard agar well-diffusion method (Bajpai et al., 2016b). All assays were performed in triplicate.

3.5 Antioxidant activity (tertiary screen)

The capacity of scavenging α-α-diphenyl-β-picrylhydrazyl (DPPH) radical of the candidate LAB was determined using the method explained by Lin et al., (2018). The Fenton reaction method was used for the assessment of hydroxyl radical scavenging test (Li et al., 2012). All tests were repeated three times.

3.6 Stress tolerance (tertiary screen)

The stress tolerance of the candidate LAB was determined using the method reported by Kobierecka et al., (2017); Wang et al., (2018b) with minor modifications. The survival of each LAB candidate was evaluated under different conditions. Briefly, the strains were grown overnight in MRS broth at 37 ℃, and then transferred to fresh MRS broth (1:100 inoculum, v/v) within the following conditions for subculture: (a) adjusted to pH 6.2, pH 5.0, pH 4.0, pH 3.0 and pH 2.0 (with 1 M HCl), the control was broth without HCl, (b) adjusted to 0.1%, 0.2%, 0.3%, 0.4% and 0.5% (w/v) of bile salts (Sigma-Aldrich, St. Louis, MO, USA) with no pH adjustment, broth without bile salts was used as the control. All candidates were cultured at 37 ℃ for 4 h, the viable cells were counted by the plate count method and the results were expressed as survival rate. All tests were repeated four times.

3.7 Morphological, biochemical, and molecular characteristics of LC-9-1

The characteristics of LC-9-1 were determined using the method explained by Bajpai et al., (2016a) with minor modifications. Firstly, the characteristic colonies were Gram stained, and bacteria were examined for morphology under the microscope (Olympus CX40, Olympus Optical Co. Ltd., Hamburg, Germany) and a scanning electron microscopic (SEM, Inspect F50, FEI Company, Hillsboro, OR, USA) (Bajpai et al., 2016b). A growth curve assay of LC-9-1 was performed with a previously described method (Fitzgerald et al., 2020). 16S rRNA gene sequencing and phylogenetic analysis were adopted to specify the molecular characteristics of LC-9-1. Briefly, genomic DNA was isolated from LC-9-1 and then the 16S rRNA gene was amplified by PCR using the universal bacterial primers (Zhang et al., 2016), 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') with the details of the procedure: 5 min at 95 ℃ for pre-deformation, 94 ℃ for 30 s, 57 ℃ for 30 s, 72 ℃ for 90 s for 30 cycles, and 72 ℃ for 8 min as the final step. The PCR products were sequenced by the Sangon Biotech Co., Ltd. (Shanghai, China). The homologies between the obtained gene sequences and those in GenBank were evaluated using BLAST analysis on the National Center for Biotechnology Information (NCBI). A bootstrap phylogenetic tree was constructed by the neighbor-joining method, using MEGA 7 software (www.megasoftware.net accessed on 15 March 2022). Furthermore, biochemical characteristics of LC-9-1 were performed using the API 50 CHL system (API 50 CHL, BioMerieux, Lyon, France) according to the instructions from the manufacturer, a standard strain of Pediococcus acidilactici ATCC 8042 (ATCC, Manassas, VA, USA) was used as a control in the test.

3.8 Safety evaluation *in vitro* of LC-9-1

3.8.1. Hemolytic activity assay

The hemolytic activity of LC-9-1 was determined using the method explained by Maragkoudakis et al., (2006) with minor modifications. Briefly, LC-9-1 was cultured in MRS broth overnight, and then inoculated in pre-made blood agar (Beijing Land Bridge Technology Co., Ltd., Beijing, China) containing 5% (v/v)

sheep blood. The presence of a hemolysis zone was observed following incubation at 37 ℃ for 24 h.

3.8.2. Antibiotic susceptibility

Antibiotic susceptibility of LC-9-1 was determined using the agar diffusion method of CLSI, (2015) with minor modifications. Briefly, LC-9-1 was cultured in MRS broth at 37 ℃ for 18 h, and then preparing a suspension in accordance with two McFarland's scales (108 CFU/mL) (Reuben et al., 2019). A total of 100 μL suspension was spread onto MRS agar plate, followed by placement of antibiotic discs. The commercially antibiotic discs (HANGWEI, Hangzhou, China) contains Cefperazone (75 μg), Ceftriaxone (30 μg), Ceftazidime (30 μg), Cefuroxime (30 μg), Cefradine (30 μg), Cefazolin (30 μg), Cefalexin (30 μg), Minocyline (30 μg), Doxycycline (30 μg), Tetracycline (30 μg), Ciprofloxacin (5 μg), Clindamycin (2 μg), Erythromycin (15 μg), Neomycin (30 μg), Kanamycin (30 μg), Gentamicin (10 μg), Amikacin (30 μg), Vancomycin (30 μg), Piperacillin (100 μg), Ampicillin (100 μg), Oxacillin (1 μg), Penicillin (10 μg), Chloramphenicol (30 μg), Furazolidone (300 μg). Plates were incubated for 24 h at 37 ℃ and the diameters of the clear zones were measured and classified as sensitive (S), intermediate (I), and resistance (R) according to the guidelines for CLSI.

3.9 *In vivo* testing

3.9.1 Strain preparation

The Pediococcus acidilactici LC-9-1 was emulsified into microcapsules (prepared by Challenge Biotechnology Co., LTD (Beijing, China, viable count $\geq 5.0 \times 10^{10}$ CFU/g). Following a conservative strategy, the amount of LC-9-1 in feed was examined daily throughout the experiment in order to ensure cell viability (Nikoskelainen et al., 2003).

3.9.2 Experimental design and bird management

The trial was conducted at the Nankou pilot base of the Chinese Academy of Agricultural Sciences. The animal experiments were managed according to the National Institute of Animal Health approved protocol, and research reporting followed the guidelines of ARRIVE (Kilkenny et al., 2012).

A total of 120 one-day-old male Arbor Acres (AA) broilers (body weight, 38.4 ± 0.7 g) were randomly allocated into 2 treatment groups with 6 replicates of 10 birds each. The control (CON) group was fed a basal diet, which met the nutritional requirements of broilers (Supplementary Table S1). The PA group was fed a basal diet containing 200 mg/kg LC-9-1 (5.5×10^9 CFU/kg). The experiment lasted for 42 days in two feeding phases, starter (1–21 d) and grower (22–42 d), and all the broilers were housed in the same environmentally controlled facility (cleaning cage equipped with the fiberglass feeders and plastic net floor). All birds were allowed feed and water ad libitum, and given the same photoperiod (16

h light: 8 h dark), relative humidity (1–7 d, 60–70%; 8–42 d, 50–60%) and room temperature (1–7 d, 33 ± 2 °C; 8–16 d decreased stepwise to 24 °C; 17–42 d 24 °C). The excreta were cleared daily. Broilers were subjected a routine vaccination program, and their health was monitored daily.

3.9.3 Sampling

Body weight (BW) and feed consumption were measured at 21 and 42 d of age, average daily gain (ADG), average daily feed intake (ADFI) and the feed intake/weight gain (F/G) ratio were calculated for the different phases. At 21 and 42 d of age, one broiler (close to the average BW) from each replicate was selected after a 12 h fasting. Blood samples (2.5 mL) were collected from the wing vein into EDTA-containing and anticoagulant-free vacuum test tubes (5 mL), respectively, and immediately placed on ice. Serum was harvested from nonanticoagulated whole blood by centrifuging at 3000× g for 10 min, and stored at −20 °C until analyzed. The slaughter performance and immune organ indexes were measured according to the production performance noun terms and metric statistics method of poultry (NY/T823-2004) (Liu et al., 2021; Gao et al., 2022c). Liver, spleen and intestinal tissues (jejunum and ileum) were collected and fixed in 10% buffered formaldehyde (pH 7.4) for histological analysis. At 42 d of age, the ileal contents of 6 broilers were collected and snap-frozen in liquid nitrogen, followed by storage at −80 °C for DNA extraction.

3.9.4 Hematological and serum biochemical indexes analysis

The red blood cell count (RBC), hemoglobin concentration (HGB), white blood cell count (WBC), and lymphocytes (Lym) were measured with an auto hematology analyzer (XT-1800i, Sysmex Corporation, Tokyo, Japan). The alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured using an automatic blood biochemical analyzer (AU640, Olympus Corporation, Tokyo, Japan). The total protein (TP) and albumin levels of serum were measured using commercial assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) using a colorimetric method. The globulin content was obtained by subtracting the albumin value from that of the TP (Salem et al., 2018). Total antioxidant capacity (T-AOC), activity of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and malonaldehyde (MDA) concentrations in the serum were determined by the commercial assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) with an automated fluorescence instrument (MultiskanM™ SkyHigh, Thermo Fisher Scientific, Waltham, MA, USA).

3.9.5 Histological analysis

Fixed intestine samples were dehydrated with increasing concentrations of ethyl alcohol (75%, 85%, 95%, and 100%), cleared in xylene, and embedded in paraffin. Five-micrometer-thick sections were prepared using a microtome, then the paraffin-embedded sections were deparaffinized, dehydrated and stained by hematoxylin–eosin (H&E). Visualization was performed under a light microscope

(Olympus CX40, Olympus Optical Co. Ltd., Hamburg, Germany). For each section, tissue was observed at a magnification of ×40, gross lesions were observed, and the images were taken at magnifications of ×100 and ×400 (only for specific lesions). Epithelial thickness, villus length and crypt depth were measured at least 10 well-oriented villi, and the villus length/crypt depth ratio (V/C) ratio was calculated. Goblet cells were identified by periodic acid-Schiff (PAS) staining according to previously described method (Wang et al., 2021a). Target cells were stained purple and were counted as the number of cells per 100 μm of the villi.

3.9.6 Microbial analysis

Microbial genomic DNA of the intestinal contents was extracted under sterile conditions using the QIAamp DNA stool Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions, and then the DNA integrity and purity were assessed by agarose gel electrophoresis and NanoDrop2000 (Thermo Fisher Scientific, Waltham, MA, USA), respectively. Subsequently, gene sequencing was implemented by Majorbio Biotech Co., Ltd. (Shanghai, China). The V3 to V4 variable region of the 16S rRNA gene was amplified with universal primers 338F and 806R. The PCR products were collected and sequenced using the Illumina MiSeq platform (Illumina, Madison, WI, USA). High-quality reads were filtered and clustered into operational taxonomic units (OTUs) based on sequences with ≥97% similarity and then analyzed using the QIIME software (version 1.9.1). The online platform (https://cloud.majorbio.com/ accessed on 3 June 2022.) of Majorbio Biotech Co., Ltd. was used to analyze the reads data. In particular, alpha-diversity indices including Chao1 index, Shannon index, Coverage index and numbers of OUTs were analyzed by Student's t-test at OTU level. The beta-diversity analysis includes the principal component analysis (PCA) and principal coordinate analysis (PCoA) (Lozupone and Knight, 2005). The Kruskal–Wallis rank sum test was employed for analysis of the relative abundance at the phylum and genus levels. Linear discriminant analysis (LDA) effect size (LEfSe) analysis was implemented using the non-parametric factoria Kruskal–Wallis rank sum test to obtain significantly different species between the CON and PA group (Segata et al., 2011), differences between groups were assessed using the Wilcoxon rank sum test, and finally LDA was used to access the influence of each species abundance on the differences.

3.10 Statistical analysis

The data were analyzed by a one-factor ANOVA procedure using SPSS 19.0 software package for Windows (SPSS Inc., Chicago, IL, USA). Significant differences between groups were separated using Duncan's multiple range test. Differences were considered significant at $p < 0.05$. The graphs were designed using GraphPad Prism 9 Project (GraphPad Software Inc., San Diego, CA, USA) and Origin 8.5 (Origin Lab, Berkeley, CA, USA).

4. Results

A total of 237 potential LAB isolates were isolated, and the workflow is given in Figure 12.

4.1 Primary screen

In the primary screen, potential strains with strong acid-producing capacity were selected by culturing at 30 ℃ and 39 ℃. The best 30 isolates in terms of their acid-producing potential are shown in Supplementary Figure S1, of which 21 strains appeared in both datasets and were further characterized.

4.2 Secondary screen

After primary screening, the most promising strains of LAB were tested for antimicrobial activity against four pathogenic microorganisms. The well-diffusion test resulted in the isolation of 10 strains with the strongest inhibitory effects of different pathogens (Supplementary Figure S2), among which four isolates (LC-2-5, LC-2-9, LC-3-9 and LC-9-1) were found to be the most prevalent strains and were selected for further analysis.

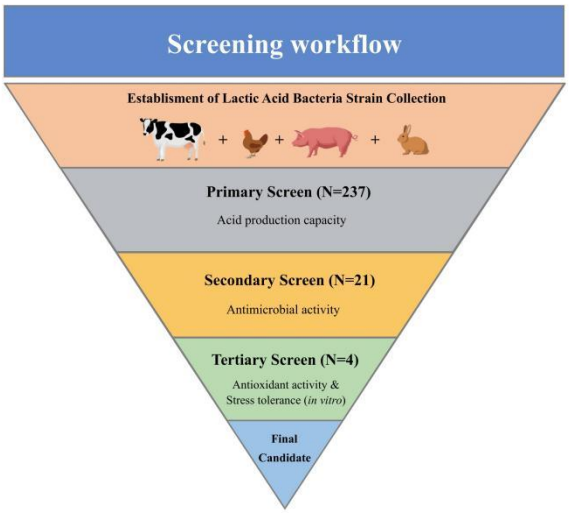

Figure 12. Workflow of the screening. The process was divided into three parts that were performed to narrow down the total of 237 potential LAB isolates to the one strain with the highest observed antibacterial and antioxidative activity.

4.3 Tertiary screen

As shown in Figure 13A, the scavenging effects of candidates on DPPH radicals ranged from 5.66% to 27.99%. LC-9-1 and LC-3-9 exhibited the highest removal efficiency, with no significant difference between the two strains. Figure 13B shows the hydroxyl radical scavenging activity of the four strains, which ranged from 9.82% to 36.42%. The efficiency of LC-9-1 was significantly higher than that of the other strains, with a scavenging activity of 36.42% ($p < 0.05$).

The tolerance of the strains to acid/alkaline conditions is shown in Figure 13C, with almost all of them demonstrating proliferation inhibition at pH below 5. The survival rates of the strains were significantly different after being treated at pH 2.0 ($p < 0.05$). LC-9-1 had the highest survival rate of 61.96%, followed by LC-3-9, the lowest were LC-2-5 and LC-2-9. The results of tolerance to bile salts are showed in Figure 13D. Overall, the survival rates of the four hit strains were similar when treated with 0.1% and 0.2% level bile salts, then declined with bile salt level. The survival rates of LC-3-9, LC-2-5 and LC-2-9 were all below 40% under 0.5% bile salt conditions, while the most tolerant stain was LC-9-1, with a survival rate of 46.01%; thus, it was chosen for the subsequent trials.

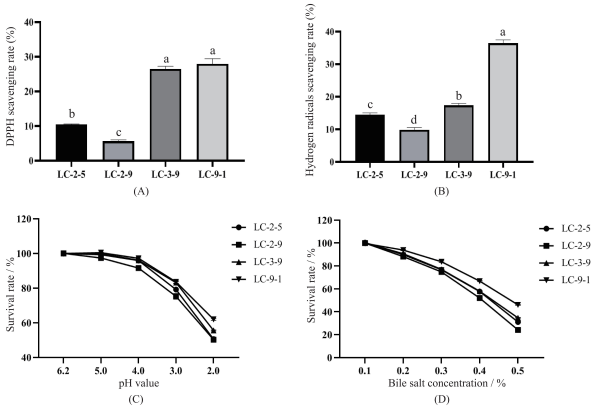

Figure 13. Antioxidant activity and stress tolerance of the candidate LAB. (A) DPPH scavenging activity; (B) hydroxyl radical scavenging activity; (C) tolerance of the strains to different pH treatments; (D) tolerance of the strains to different concentrations of bile salts.

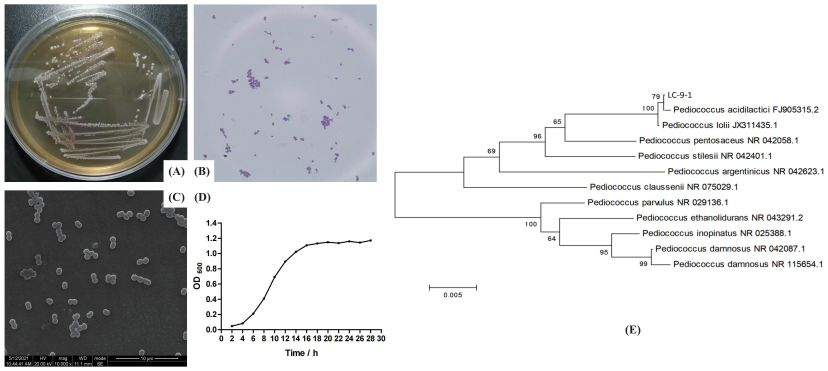

Figure 14. Morphological, biochemical, and molecular characteristics of LC-9-1. (A) Colony morphology; (B) Gram stain showing Gram-positive cocci; (C) SEM image of LC-9-1 showing a spherical morphology (×1000); (D) growth curve of LC-9-1; (E) phylogenetic tree analysis of LC-9-1.

4.4 Morphological, biochemical, and molecular characteristics of LC-9-1

The isolate of LC-9-1 appeared as creamy white, opaque, round and small colonies on the surface of MRS agar of the isolate LC-9-1, which was confirmed to be Gram-positive cocci (spherical shaped) by microscopic evaluation (Figure 14A and 14B). The cells tended to occur in pairs (Figure 14C). The proliferation curves appeared in a typically sigmoidal shape, consisting of a latency phase (0–4 h), a logarithmic phase (4–16 h) and a plateau phase (16 h later) (Figure 14D). According to the 16S rRNA gene sequencing and phylogenetic characteristics, the similarity of the LC-9-1 strain to *Pediococcus* spp. was 99.9% (Figure 14E); thus, the strain was preliminarily identified as *P. acidilactici*. Biochemical analysis was performed using the API 50 CHL strip kit and the strip capsules turned yellow, indicating a complete fermentation of sugar by strain. The API web software confirmed that LC-9-1 utilized carbohydrates including Salicin, D-cellobiose, D-ribose, D-xylose, D-saccharose, D-trehalose, D-galactose, D-glucose, D-fructose, D-mannose, Gentiobiose, D-tagatose, N-acetylglucosamine, Amygdalin, Arbutin and Esculin (Supplementary Table S2). The strain exhibited no difference in color change compared to the standard strain of P. acidilactici ATCC 8042, suggesting that both strains had the same fermentation of sugar pathways. Taken together, LC-9-1 was identified as *P. acidilactici* and has been deposited in the China General Microbiological Culture Collection Center (CGMCC, Beijing, China), with the patent number (CGMCC No. 21345).

4.5 Safety evaluation in vitro of LC-9-1

The hemolytic activity of LC-9-1 was judged by observing the hemolytic rings on blood agar plates after a 24 h incubation. The strain was not involved in the lysis of erythrocytes (results not shown). Supplementary Table S3 shows the antibiotic susceptibility profile of the LC-9-1, validating that it was susceptible to most antibiotics. Specifically, the strain was extremely susceptible to Cefoperazone, Ceftriaxone, Erythromycin, Cefuroxime, Cefradine, Cefalexin, Minocyline, Doxycycline, Tetracycline, Piperacillin, Ampicillin, Penicillin, Chloramphenicol, Furazolidone and Clindamycin. Additionally, it was found that LC-9-1 was completely resistant to Kanamycin, Gentamicin, Amikacin and Ciprofloxacin.

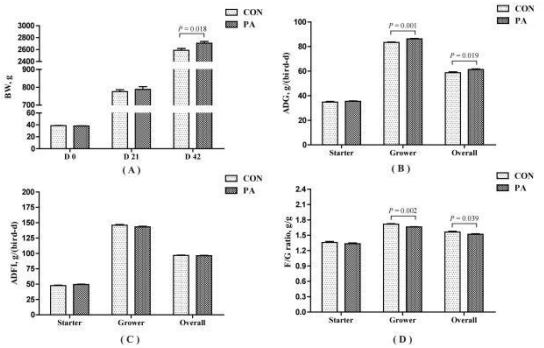

Figure 15. Effect of LC-9-1 on growth performance of broilers (*n*=6). CON=control group, broilers were fed a corn–soybean basal diet, PA = *P. acidilactici* group, broilers were fed a basal diet containing 5.5×10^9 CFU/kg LC-9-1. BW, body weight; ADG, average daily gain; ADFI, average daily feed intake; F/G ratio = feed intake (g)/weight gain (g). The values with superscript (*) were significantly different, * $0.01 < p < 0.05$; ** $p \leq 0.01$.

4.6 In vivo testing

4.6.1. Growth performance

The average mortality rate was 0.5% during the experiment (data not presented), and there were no significant differences between the groups. The response of growth performance is shown in Figure 15. Compared with the CON group, LC-9-1 significantly increased the BW of broilers at 42 d of age and the ADG during the grower and overall periods ($p < 0.05$), and decreased the F/G ratio during the grower and overall periods ($p < 0.05$). No significant differences were found in the ADFI.

Table 10. Effect of LC-9-1 on slaughter performance of broilers at day 42 (*n*=6)

Items	CON	PA	SEM	*p*-value
Dressing percentage, %	89.55	90.53	0.471	0.320
Half-eviscerated yield, %	84.86	86.30	0.457	0.117
Eviscerated yield, %	75.13	75.63	0.484	0.628
Breast muscle ratio, %	13.67	13.63	0.231	0.963
Thigh muscle ratio, %	10.99	10.93	0.353	0.934
Abdominal fat ratio, %	2.22**	1.76	0.083	0.001

CON=control group, broilers were fed a corn-soybean basal diet, PA=*P. acidilactic*i group, broilers were fed a basal diet containing 5.5×10^9 CFU/kg LC-9-1. SEM=standard error of means; The values having superscript (*) in the same row were significantly different, * $0.01 < p < 0.05$; ** $p \leq 0.01$.

4.6.2 Slaughter performance and immune organ indexes

The effect of LC-9-1 on slaughter performance of broilers is listed in Table 10. Dietary supplementation with LC-9-1 did not have a negative effect on the relevant parameters. However, the abdominal fat rate of broilers in PA group was significantly lower than that in CON group ($p < 0.01$). In addition, no effect was observed on immune organ indexes of broilers at 21 and 42 d of age (Table 11).

Table 11. Effect of LC-9-1 on immune organ indexes of broilers (*n*=6)

Items	Dietary treatment		SEM	*p*-value
	CON	PA		
Day 21				
Spleen index, mg/g	0.88	0.99	0.040	0.198
Thymus index, mg/g	2.85	2.84	0.017	0.784
Bursa of fabricius index, mg/g	1.96	1.98	0.011	0.387
Day 42				
Spleen index, mg/g	1.17	1.22	0.016	0.111
Thymus index, mg/g	2.17	2.19	0.013	0.397
Bursa of fabricius index, mg/g	1.04	1.04	0.019	0.901

CON=control group, broilers were fed a corn-soybean basal diet, PA=*P. acidilactic*i group, broilers were fed a basal diet containing 5.5×10^9 CFU/kg LC-9-1. Spleen index, thymus index and bursa of fabricius index were calculated as follows: (1) Spleen index (mg/g)=spleen weight (mg)/body weight (mg), (2) Thymus index (mg/g)=thymus weight (mg)/body weight (g), (3) Bursa of fabricius index (mg/g)=bursa of fabricius (mg)/body weight (g); SEM=standard error of means; The values having superscript (*) in the same row were significantly different, * $0.01 < p < 0.05$; ** $p \leq 0.01$.

4.6.3 Hematological and serum biochemical index analysis

As shown in Table 12, compared with the CON group, LC-9-1 treatment did not affect the hematological or serum biochemical indexes of birds at 21 d of age. However, at 42 d of age, LC-9-1 treatment significantly decreased ALT and AST and elevated A/G ratio compared to the CON group ($p < 0.05$).

Table 12. Effect of LC-9-1 on hematological and serum biochemical indexes of broilers (*n*=6)

Items	Dietary treatment		SEM	*p*-value
	CON	PA		
Day 21				
RBC, $\times10^{12}$/L	2.42	2.48	0.051	0.569
HGB, g/L	100.83	103.67	2.041	0.592

WBC, $\times 10^9$/L	144.90	140.95	1.290	0.130
LYM, $\times 10^9$/L	52.81	50.01	1.270	0.292
ALT, U/L	2.22	2.10	0.031	0.054
AST, U/L	239.73	230.36	2.465	0.051
TP, g/L	3.17	3.21	0.024	0.377
Albumin, g/L	1.38	1.41	0.013	0.248
Globulin, g/L	1.79	1.80	0.013	0.636
A/G ratio	0.77	0.79	0.005	0.286
Day 42				
RBC, $\times 10^{12}$/L	2.62	2.44	0.054	0.112
HGB, g/L	105.17	105.33	2.136	0.971
WBC, $\times 10^9$/L	143.99	140.17	1.228	0.123
LYM, $\times 10^9$/L	56.92	56.74	0.831	0.920
ALT, U/L	3.22**	3.00	0.038	0.001
AST, U/L	244.10**	228.01	3.040	0.002
TP, g/L	3.04	3.01	0.027	0.628
Albumin, g/L	1.30	1.33	0.016	0.257
Globulin, g/L	1.74	1.67	0.018	0.068
A/G ratio	0.75	0.80**	0.010	0.003

CON=control group, broilers were fed a corn-soybean basal diet, PA=*P. acidilactic*i group, broilers were fed a basal diet containing 5.5×10^9 CFU/kg LC-9-1. RBC, red blood cell count; HGB, hemoglobin concentration; WBC, white blood cell count; Lym, lymphocytes; ALT, alanine aminotransferase; AST, aspartate aminotransferase; TP, total protein; A/G ratio=albumin/globulin; SEM=standard error of means; The values having superscript (*) in the same row were significantly different, * $0.01 < p < 0.05$; ** $p \leq 0.01$.

4.6.4 Serum antioxidant performance

The effect of LC-9-1 on serum antioxidant of broilers is shown in Table 13. Feeding with LC-9-1 significantly increased the activities of T-AOC and SOD at 21 and 42 d of age compared to the basal diet ($p < 0.05$). Other than that, LC-9-1 supplementation significantly decreased the MDA concentration at 21 d of age ($p < 0.05$). Nevertheless, no significant difference was found between the two groups regarding the GSH-Px activity at 21 and 42 d of age.

Table 13. Effect of LC-9-1 on serum antioxidant performance of broilers (*n*=6)

Items	Dietary treatment		SEM	*p*-value
	CON	PA		

Day 21				
T-AOC, U/mL	8.36	8.58*	0.048	0.017
SOD, U/mL	104.85	112.65*	1.521	0.003
GSH-Px, U/mL	214.83	228.83	2.351	0.088
MDA, nmol/mL	4.78**	4.34	0.077	0.001
Day 42				
T-AOC, U/mL	9.30	9.40*	0.022	0.014
SOD, U/mL	129.00	147.50**	3.610	0.003
GSH-Px, U/mL	242.83	252.50	3.227	0.141
MDA, nmol/mL	5.30	5.17	0.040	0.082

CON=control group, broilers were fed a corn-soybean basal diet, PA=*P. acidilactic*i group, broilers were fed a basal diet containing 5.5×10^9 CFU/kg LC-9-1. T-AOC, total anti-oxidant capacity; SOD, superoxide dismutase; GSH-Px, glutathione peroxidase; MDA, malonaldehyde; SEM=standard error of means; The values having superscript (*) in the same row were significantly different, * $0.01 < p < 0.05$; ** $p \leq 0.01$.

4.6.5 Histological analysis

The effect of LC-9-1 on the intestinal histomorphology of broilers is shown in Table 14. At 21 d of age, there were no significant differences in the histomorphometric features of the jejunum and ileum between the CON and PA groups. At 42 d of age, the villus length and V/C ratio in the ileum were significantly higher in the PA group than in the CON group ($p < 0.05$). However, LC-9-1 did not affect the epithelial thickness of jejunum and ileum at 21 and 42 d of age. The number of goblet cells in the ileal villus were significantly increased in the LC-9-1 group at 21 and 42 d of age ($p < 0.05$, Figure 16).

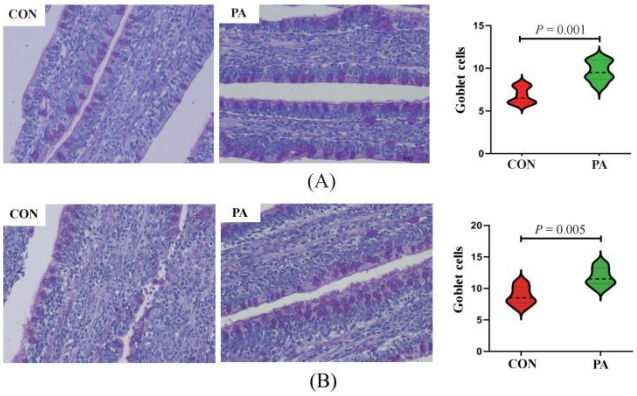

Figure 16. Effect of LC-9-1 on ileal epithelial goblet cells in broilers at 21 and 42 d of age (*n* = 6). CON = control group, broilers were fed a corn–soybean basal diet, PA = *P. acidilactici* group, broilers were fed a basal diet containing 5.5 × 10^9 CFU/kg LC-9-1. (A)&(B) Representative images of ileal PAS stained (400×) and statistical analysis of the number of epithelial goblet cells at 21 and 42 d of age, respectively.

Table 14. Effect of LC-9-1 on histomorphology of the small intestinal sections in broilers (*n*=6)

Items	Intestine	Treatment		SEM	*p*-value
		CON	PA		
Day 21					
Jejunum	Villus length, μm	1310.05	1336.47	21.775	0.569
	Crypt depth, μm	182.32	185.83	3.672	0.654
	V/C ratio	7.20	7.25	0.205	0.914
	Epithelial thickness, μm	199.15	213.10	4.415	0.117
Ileum	Villus length, μm	869.15	908.50	16.191	0.241
	Crypt depth, μm	206.20	200.14	4.888	0.561
	V/C ratio	4.24	4.56	0.121	0.205
	Epithelial thickness, μm	219.77	231.23	6.354	0.392
Day 42					
Jejunum	Villus length, μm	1338.93	1478.31	41.007	0.088
	Crypt depth, μm	239.87	234.10	1.749	0.100
	V/C ratio	5.59	6.32	0.198	0.063
	Epithelial thickness, μm	234.83	246.49	6.461	0.392

Ileum	Villus length, μm	842.92	1030.13**	31.481	＜0.001
	Crypt depth, μm	219.55	212.67	3.880	0.401
	V/C ratio	3.86	4.85**	0.175	＜0.001
	Epithelial thickness, μm	349.36	342.28	7.313	0.651

CON=control group, broilers were fed a corn-soybean basal diet, PA=*P. acidilactic*i group, broilers were fed a basal diet containing 5.5×10^9 CFU/kg LC-9-1. V/C ratio=Villus length (μm)/Crypt depth (μm). SEM=standard error of means. The values having superscript (*) in the same row were significantly different, * $0.01 < p < 0.05$; ** $p \leq 0.01$.

4.6.6 Microbial analysis

To understand whether LC-9-1 could reshape the gut microbiota community, we investigated the change in the ileal microbiota composition (Figure 17). The results revealed that the Chao1 index of OTU level was not significantly different between the groups (Figure 17A), while the Shannon index of OTU level was significantly higher in the PA broilers ($p < 0.05$) (Figure 17B), indicating the treatment of LC-9-1 caused a certain degree of change in the intestinal microbiota diversity. The coverage index for every sample was greater than 0.997 (Figure 17C), indicating that the subsequent analyses were not affected by biases in sequencing depth. Furthermore, an increasing trend in the number of OTUs was observed in the PA group compared with the CON group (Figure 17D). The β-diversity analysis is shown in Figure 17E&17F, and the results of PCA and PCoA showed that samples in the CON and PA groups had different community compositions and structures, suggesting that a significant segregation had occurred between microbiota groups. The stacked bar graphs were created to show the different OTUs at the level of Phylum and Genus (Figure 17G&17H). Regarding the results indicating the quantity of OTUs at the phylum level, five bacteria had an average relative abundance >1% for each group, of which the most abundant in the CON group were Firmicutes and Proteobacteria, while in the PA group, the most abundant were Firmicutes and Cyanobacteria. At the genus level, a total number of eight bacteria had relative abundances above 1%, of which the most abundant in the CON were Lactobacillus, *Enterococcus* and *Escherichia-Shigella*, while in the PA group the most abundant were *Enterococcu*s, *Lactobacillus* and *Chloroplast*. Additionally, LEfSe analysis (LDA > 2) revealed significant differences in microbiota structure between the CON group and the PA group (Figure 17I). Furthermore, adopting the Kruskal–Wallis rank sum test, we found that Cyanobacteria and Actinobacteriota at the Phylum level were more abundant ($p < 0.05$) in the PA group than in the CON group (Figure 17J), while the abundance of Proteobacteria was significantly decreased ($p < 0.05$). At the genus level (Figure 17K), 15 bacteria were significantly different, the relative abundance of *Escherichia-Shigella*, *Romboutsia* and *Macrococcus* were notably decreased in the PA group ($p < 0.05$), while other species of bacteria such as *Chloroplast*, *Rothia* and *Ruminococcus* were significantly elevated ($p < 0.05$). Importantly, we also found a significant increase in the relative abundance of *Pediococcus* in the PA group ($p < 0.01$).

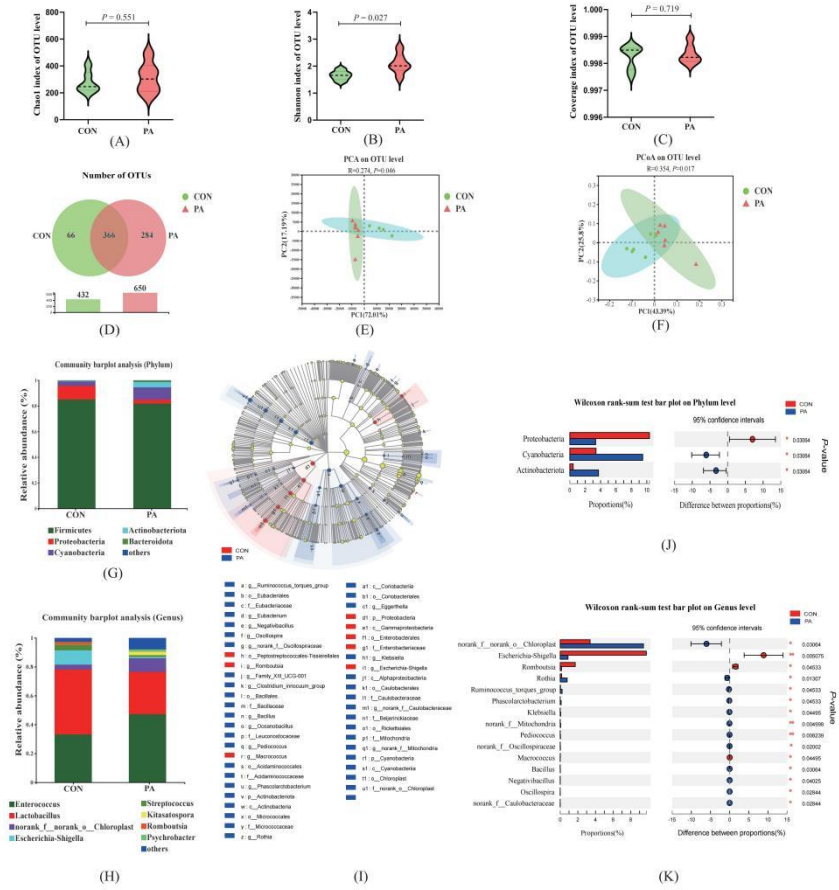

Figure 17. Effect of LC-9-1 on gut microbiota in broilers at 42 d of age (*n*=6). A, Chao1 index of OUT level; B, Shannon index of OUT level; C, Coverage index of OUT level; D, Number of OTUs; E&F, β-diversity was estimated by the PCA (E) and PCOA (F) on OUT level; G&H, The relative abundance of bacteria, G at the phylumand level and H at genus level; I, Cladogram of LEfSe multi-level species difference discriminant analysis (LDA > 2), different color nodes indicate microbial communities that are significantly enriched in the corresponding groups and significantly different between groups; J&K, Comparative analysis of the relative abundance of bacteria, J at the phylum level and K at genus level.

5. Discussion

LABs are recognized as an excellent probiotic that produces organic acids, bacteriocin, and also have excellent antioxidative properties (Huang et al., 2018; Lin et al., 2018). The bacteria decompose carbohydrates into organic acids during fermentation, which play an important role in improving the nutrition and storage quality of food (Abbasiliasi et al., 2017). In addition, it has been shown that organic acids could improve the innate immunity of poultry (Ragaa and Korany, 2016). Based on this, the acid production efficiency of LAB was used as an evaluation index in the primary screen. The antibacterial activity of LAB was determined by secreting different antibacterial substances, including antibacterial peptides, bacteriocins, hydrogen peroxide, alcohol, organic acids, etc. According to Reuben's report, organic acids and small molecular substances were the most promising components (Reuben et al., 2019), which is another factor recommending acid-producing efficiency as the primary screening criterion in our study. Furthermore, the antibacterial effects of screened LABs were tested against *E. coli*, *Salmonella*, *S. aureus* and *P. mirabilis*, which are the most common pathogenic species in poultry infections (Zhou et al., 2020a; Bao et al., 2021; Rostami et al., 2021; Li et al., 2022c). The most widely used commercial rearing system at present is closely related to oxidative stress in broilers (da Silva Frasao et al., 2021). Serious oxidative stress can lead to inferior growth performance, tissues damage and even death (Chen et al., 2021). A variety of LAB have been shown to have antioxidant activity, and antioxidant enzymes have been proven to be important defense systems against oxidative stress, such as lipoteichoic acid, exopolysaccharides and cell-surface proteins (Lin et al., 2018). In tertiary screening, the scavenging ability of LAB on DPPH free radicals and hydroxyl radical were taken as an evaluation index, and the performance of potential probiotics in terms of acid resistance and bile salt tolerance is also important for their survival and growth in the intestinal tract (Ehrmann et al., 2002). Finally, the LC-9-1 strain showed the best performance among the candidates. According to its morphological, biochemical and molecular characteristics, we finally identified the target strain as *P. acidilactici*. Although the majority of LAB are generally recognized as safe (Köberl et al., 2019). A safety evaluation of LC-9-1 was also undertaken. The strain was not involved in the lysis of erythrocytes, and it was validated as being susceptible to most antibiotics. Furthermore, LC-9-1 was found to be resistant to aminoglycoside antibiotics (kanamycin, gentamicin, amikacin) and quinolone antibiotics (ciprofloxacin). It is worth noting that many species of LAB are intrinsically resistant to antibiotics, such as kanamycin, gentamicin, vancomycin, ciprofloxacin and streptomycin, but the antibiotic resistance genes could not be horizontally transmitted between different bacterial communities (Wang et al., 2018b; Campedelli et al., 2019). These results illustrate that LC-9-1 has potential as a safe probiotic candidate.

In vitro assessment may not be representative of the situation in vivo; therefore, we evaluated the safety and application potential of the LC-9-1 by adding it to broiler diets. The results showed that 5.5×10^9 CFU/kg of LC-9-1 increased the body weight at 42 d of age and increased the ADG during the grower and overall

periods of broilers. Previous studies have shown that *P. acidilactici* combined with xylan oligosaccharides and mannan-oligosaccharides, respectively, could benefit the intestinal health and growth performance of broilers, through possible mechanisms including improving intestinal morphology, optimizing the intestinal microflora structure, and reducing the relative abundance of pathogenic microorganisms (Jazi et al., 2018; Wu et al., 2021). Moreover, the data showed that dietary LC-9-1 supplementation not only had no negative effects on slaughter performance and immune organ indexes, but also significantly reduced abdominal fat in broilers. With the constant improvement in the growth speed of broilers, the problem of excessive abdominal fat deposition is becoming more prominent. It has been reported that excessive fat is associated with decreased immunity and the occurrence of various diseases (Chen et al., 2019). Excessive abdominal fat not only reduces the immunity of broilers to diseases, it also reduces the economic value of the broilers. Therefore, reducing broilers' abdominal fat deposition has become one of the main tasks for researchers in the broiler industry.

Broilers are often affected by various stressors under the conditions of intensified and industrialized modes of production, which directly or indirectly cause tissue damage, including in the intestinal tract and liver. The activity levels of serum ALT and AST are considered to be diagnostic tools for liver injury, and any pathological or toxic injury may result in elevated activity levels of ALT and AST (Alhidary et al., 2016). It was found in the present study that LC-9-1 reduced the activity levels of ALT and AST, indicating that the liver injury was recovered to a certain extent. This result was also verified by liver histological examination; the lesions of the PA broilers were milder than those of the CON broilers, indicating that LC-9-1 had a certain protective effect on the liver. There is a point that requires clarification. It has previously been shown that cage-rearing systems can induce an increase in serum MDA, which leads to a higher risk of tissue damage (Shields and Greger, 2013; Estévez, 2015). We consider that the CON broilers showed tissue damage that was associated with the cage rearing system applied. Moreover, only a small range lymphocyte aggregates were observed in the LC-9-1 broilers, which may be related to the antioxidant effect of LC-9-1.

Albumin level is associated with systemic inflammatory response and reflects nutritional status, and globulin level is associated with chronic inflammation (Gabay and Kushner, 1999; Li et al., 2018a). Furthermore, the decrease in A/G ratio indicated an inflammation in poultry (Lumeij, 1997). In this study, LC-9-1 significantly increased the A/G ratio compared with the CON group, and we speculated that LC-9-1 protects the physiological function of the liver and maintains the liver's supplement of serum albumin. Moreover, LC-9-1 also reduces the inflammation level of the body and maintains a normal level of globulin.

As an adaptive mechanism, broilers tend to undergo physiological changes in stressful environments. High-density feeding not only induces oxidative stress, it also impairs the immune and antioxidant systems (Son et al., 2022). In biological

systems, oxidative stress is usually caused by an imbalance between antioxidant and pro-oxidative system. Excessive generation of reactive oxygen species (ROS) causes oxidative damage to cells, and then triggers biological damage, impairing growth (Andreadis et al., 2007; Estevez and Petracci, 2019). T-AOC reflects the ability of the non-enzymatic antioxidant defense system (Giustarini et al., 2009). SOD regulates the balance of oxidation and anti-oxidation in vivo through enzymatic reactions, and it is seen as the first line of the antioxidant system by neutralizes the most unstable ROS superoxide anion to hydrogen peroxide. MDA is an end product of lipid peroxidation and is used as a biomarker of oxidative stress (Urso and Clarkson, 2003). In the present study, the increased activities of T-AOC and SOD, and the decreased MDA concentration in the PA group indicated that the LC-9-1 improves the potential of the broilers' endogenous antioxidant defense capacity. The mechanism for this may be related to the antioxidant properties of LC-9-1. Similar to our results, Lin et al. reported that supplementation with *L. plantarum* AR501 significantly increased the T-AOC activities in the mice (Lin et al., 2018). It is well known that inflammation is one of the major downstream responses to oxidative stress, and inflammation can further aggravate oxidative stress (Shen et al., 2021). This connection is increasingly evident in biological processes and cannot be ignored. For the above reasons, the resistance to pathogenic microorganisms and antioxidant activity of LAB were taken as screening principles in this study.

The absorption site of nutrients is mainly in the small intestine in broilers. The height of the villus indicates the area of intestinal absorption (Daneshmand et al., 2019). The V/C ratio represents the functional state of the intestine (Inatomi and Otomaru, 2018). Probiotics have been demonstrated to protect the integrity of the gut tissue through various mechanisms (Liew et al., 2019; Dong et al., 2020). Similar results were observed in our study, with LC-9-1 being beneficial to maintaining the morphology of the intestinal epithelium, and this provided a reasonable explanation for the improvement of growth performance in the PA group. Mucin secreted by goblet cells was primarily involved in innate host defense (Jung et al., 2018). In this study, the intestinal goblet cells of broilers in the PA group were significantly increased at 21 and 42 d of age, indicating that LC-9-1 may enhance intestinal innate immunity.

The intestinal microbiota community plays a variety of roles in nutrient absorption and metabolism, immunity, and host health (Hong et al., 2019). It has previously been shown that the diversity of gut microbes contributes to microbiome homeostasis and the resistance to pathogenic microorganisms (Konstantinov et al., 2004). In this study, dietary supplementation with LC-9-1 increased the intestinal microbial diversity (Shannon index), but did not significantly alter the microbial community richness (Chao1 index). The PCA and PCoA analyses showed significant differences in microbial communities between the PA group and CON group. The predominant bacterial phyla in the ileum included Firmicutes, Cyanobacteria, Proteobacteria and Bacteroidetes, similar to our observations (Guo et al., 2021). The most abundant phyla in the CON broilers were Firmicutes and Proteobacteria, while in the PA group the most abundant

were Firmicutes and Cyanobacteria. Proteobacteria contains a wide variety of pathogens, such as *Salmonella*, *Escherichia coli*, and *Shigella*, which could exert pathogenic effects in the intestine of chickens (Mora et al., 2010). The decrease in Proteobacteria indicated that a relatively healthy bacterial community was achieved with LC-9-1 supplementation. Furthermore, analysis at the genus level verified the above issue. Supplementation with LC-9-1 reduced the relative abundance of *Escherichia-Shigella*, which is an opportunistic pathogen and is positively correlated with a variety of intestinal infections (Yang et al., 2019a). We also found a similar reduction in *Romboutsia*, which is associated with *C. difficile* infection (Araos et al., 2018). Correspondingly, we found an increase in the abundance of some beneficial genera, such as *Rothia* and *Ruminococcus*. Most importantly, the supplemented LC-9-1 belongs to genus *Pediococcus*, which also had significantly higher relative abundance in PA broilers. Taken together, it can be concluded that LC-9-1 contributes to maintaining the microbial balance in the intestine, and promoting the growth of beneficial bacteria, while suppressing potentially pathogenic microbes.

6. Conclusions

This study showed the newly screened and characterized LAB *P. acidilactici* LC-9-1, which was isolated from the intestinal tract of different healthy animals. Specifically, the LC-9-1 had excellent acid-producing ability, antibacterial properties, antioxidant ability and stress resistance in vitro. The in vivo experiments indicated that dietary LC-9-1 supplementation improved the growth performance, reduced the abdominal fat, enhanced the antioxidant capacity, and improved the innate immunity level of intestinal tract. The mechanism may be related to the various functional metabolites produced by LC-9-1 and its influence on the intestinal microbiota. Therefore, LC-9-1 could be considered a potential strain for further utilization in DFM or probiotic starter culture.

Supplemental information

Supplemental information is available in the online version of this article.

Table S1: Analysis composition of basal diets and nutrient level (air-dry basis, %).

Table S2: Biochemical characteristics of LC-9-1 based on carbohydrate interpretation using API 50 CHL kit.

Table S3: Antibiotic susceptibility profile of potential probiotic LC-9-1.

Figure S1: Acid-producing efficiency.

Figure S2: Antimicrobial activity.

Chapter VI

Assessment of standardized ileal digestibility of amino acids and apparent metabolizable energy in broiler chickens fed fermented peanut meal

Prior to this, we have successfully screened two bacteria for the peanut meal fermentation. In this section, we will employ conventional fermentation processes to biologically treat the peanut meal. In addition to determining the physical and chemical changes, it is crucial to assess the amino acid digestibility and metabolic energy of the fermented peanut meal in broiler chickens. These experiments will not only elucidate the effects of fermentation but also provide fundamental data for formulating broiler feed.

This chapter is based on the following publication:

Shuzhen Li, **Chong Li**, Si Chen, Xiaoying Wang, Jinmei Liu, Xuejuan Deng, Huiyi Cai and Guohua Liu

Effects of Solid-State Fermentation on the Standardized Ileal Digestibility of Amino Acids and Apparent Metabolizable Energy in Peanut Meal Fed to Broiler Chickens

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Chapter VI. Effects of solid-state fermentation on the standardized ileal digestibility of amino acids and apparent metabolizable energy in peanut meal fed to broiler chickens

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Key words: fermented peanut meal, nutritive value, ileal digestibility of amino acids, metabolizable energy, protein source, broilers

1. Abstract

Peanut meal (PNM) is a byproduct of the peanut oil extraction process, but its application is seriously limited by the presence of anti-nutritional factors, imbalance in amino acid profiles, and susceptibility to mycotoxin contamination. This study was conducted to investigate the effects of solid-state fermentation on the nutritional quality of PNM, as well as the effects of PNM and fermented peanut meal (FPNM) on the ileal digestibility of amino acids and apparent metabolizable energy (AME) of broiler chickens. The results indicated that the fermentation improved the quality of PNM by increasing the crude protein, TCA-soluble protein, and L-lactic acid concentration ($p < 0.05$), and decreasing the crude fiber, phytic acid, and aflatoxin B_1 concentration ($p < 0.05$). Solid-state fermentation also increased the free amino acids level and improved the balance of hydrolyzed amino acids of PNM. A nitrogen-free diet was used to determine the loss of endogenous amino acid in birds, and the PNM or FPNM as the only protein source to formulate semi-purified diets. The result showed that feeding on FPNM resulted in higher apparent ileal digestibility (AID) and standardized ileal digestibility (SID) values of the essential amino acids of Met, Lys, Leu, and Phe ($p < 0.05$). Moreover, the AID and SID values of the non-essential amino acids of FPNM were both higher than those of PNM, except for Pro ($p < 0.05$). The AME was determined by the classic substitution method, and the results showed that fermentation had no effect on the AME value ($p > 0.05$). In conclusion, solid-state fermentation improved the nutritional value of PNM, and FPNM was a potential ingredient as an alternative protein source for broilers.

2. Introduction

Corn and soybean meal are the traditional component of poultry diets used to meet energy and protein requirements; however, the price continues to rise due to an imbalance between demand and production. Nutritional expenditure determines 50–70% of the cost of poultry production; an increase in the cost of feedstuff would lead to an increase in production costs, driving more public focus on cost-effective protein feedstuff (Van Horne, 2018). Studies have shown that many locally available ingredients can be used as soybean meal substitutes to meet the nutritional requirements of poultry, such as sunflower meal, rapeseed meal and cottonseed meal, which are commonly used (Kowalska et al., 2020; Yu et al., 2020b; Berger et al., 2021). PNM is a byproduct of peanut oil production by high temperature pressing, which is also a promising plant protein. It has a powdery or blocky morphology, with a strong peanut flavor that is comprised of 40.1–50.9% protein, 0.7–6.0% fat, 5.8–12.6% crude fiber (CF), and also rich in vitamins, minerals, and antioxidant components (polyphenols, flavone). The nitrogen-corrected metabolic energy of broiler chickens is 9.6–12.6 MJ/kg (Batal et al., 2005). Although PNM can be fed to poultry directly, the imbalance in amino acid profiles limits its use in poultry feed, such as lower levels of Lys, Thr, and Met and an excessively high Arg/Lys ratio (3.49), which is much higher than the National Research Council Nutrient Requirements of Poultry recommended ratio (1.10–1.18) (National Research Council., 1994). Studies have shown that

excessive Arg may have negative effects on animal health and productivity (Zampiga et al., 2018). Additionally, phytic acid is the main anti-nutritional factor with a content of 1.50–2.00% in PNM, and it not only binds to metal ions, but connects to amino acid residues, which affects the absorption of nutrients by the organism (Ren et al., 2017). *Aspergillus flavus* and *Aspergillus parasiticus* infections cause aflatoxin contamination in peanuts, which also limits the application of PNM in feed (Arias et al., 2018).

Solid-state fermentation is the growth of microorganisms on solid materials under controlled conditions, which can also change the chemical or physical-chemical properties of the substrate (Olukomaiya et al., 2019). Recent studies have shown that solid-state fermentation of plant protein raw materials can improve nutritional properties. For instance, the contents of harmful compounds such as glucosinolates, non-starch polysaccharides, and phytic acid in rapeseed meal can be reduced by fermentation, and the indispensable nutrients are not lost in the process (Hu et al., 2016; Vadopalas et al., 2020). Jazi et al., (2017a) also revealed CF and free cotton phenol in cottonseed meal could be degraded by fermentation. Shi et al., (2017) reported that solid-state fermentation of corn-soybean meal mixed diets reduced the concentration of anti-nutritional factors β-soybean globulin and phytic acid while increasing the content of soluble protein. Li et al., (2022b) reported the total amino acids content of fermented soybean meal increased by 2.56%, CF and trypsin inhibitor decreased by 7.56% and 67.80%, respectively, and the standardized ileal digestibility (SID) of amino acids of broiler chickens increased significantly. Regarding PNM, previous studies found that solid-state fermentation improved the nutrient composition, and also degraded aflatoxin B_1 (AFB_1) (Yang et al., 2016a; Zhou et al., 2017). Specifically, we hope to tackle the problem of imbalance in amino acid and anti-nutritional factors in PNM via solid-state fermentation, which requires the fermentation bacteria to have the capacity to hydrolyze proteins and depolymerize phytic acid. In our laboratory's previous work, *Bacillus velezensis* (*B. velezensis*) LB-Y-1 and *Pediococcus acidilactici* (*P. acidilactici*) LC-9-1 were screened out, and they have potential in this regard.

As far as we know, few investigations have been performed on the nutritional value evaluation of FPNM in broiler chickens. In order to better understand the potential application of FPNM in broiler diets, it is necessary to comprehensively evaluate the nutritional value of it. Therefore, the purpose of this study is to analyze the changes of nutritional composition of PNM after solid-state fermentation. Additionally, we hypothesize that fermentation can improve the SID of amino acid and apparent metabolizable energy (AME) of PNM.

3. Materials and methods

3.1 Preparation of FPNM

The commercial PNM was purchased from Xunda Grain and Oil Co. LTD (Puyang, Henan, China). FPNM was prepared by two-stage solid-state fermentation of PNM using strains of *B. velezensis* LB-Y-1 (3.0×10^8 CFU/mL)

and *P. acidilactici* LC-9-1 (1.0×10^8 CFU/mL). The two strains were obtained by specific screening and preserved in a China General Microbiological Culture Collection Center, numbered as CGMCC 2.1344 and CGMCC 2.1345, respectively. The fermentation was performed as described by (Wang et al., 2020) with minor modification as follows: (1) The pretreatment of raw material: the PNM was crushed and passed through a 0.88 mm sieve, and was sterilized at 121 ℃ for 15 min, and cooled down to room temperature (26 ℃); (2) First-stage: the fermentation was performed by inoculating 6.0×10^9 CFU/kg PNM of the LB-Y-1 inoculum, and adding distilled water to make the moisture content reach 37.0%. Transferred the fully mixed PNM to the fermentation box (40 × 20 × 10 cm), with the depth of material of 5 cm and a sterile membrane was added to the fermentation box. The PNM were fermented for 54 h at 38 ℃ in quasi-aerobic conditions, remixed every 4 h; (3) Second-stage: the fermentation was performed by inoculating 2.0×10^9 CFU/kg PNM of the LC-9-1 inoculum, and adding distilled water to make the moisture content reach 40.0%. Mixed PNM were transferred into a polyethylene bag with a one-way valve (to permit carbon dioxide release during fermentation), and sealed immediately, fermented for 18 h at 37 ℃ in quasi-anaerobic conditions; (4) The fermented samples were dried at 48 ℃ after fermentation till the moisture content was 10%, and then the dried samples were ground to pass through a 0.88 mm sieve and stored at room temperature (26 ℃).

3.2 Sample preparation, chemical analysis and bacterial quantity measurements

The analysis of routine nutrients followed the guidelines of the Association of Official Analytical Chemists (AOAC). Samples were dried at 105 ℃ for 5 h in a drying oven to determine the content of dry matter (DM) (method 934.01, AOAC 2006). The total nitrogen content was determined with a combustion analyzer (Dumatherm, Gerhardt, Germany). Crude protein (CP) was calculated as N × 6.25. The ash content was determined by placing samples in a temperature-controlled furnace preheated to 600 ℃ for 2 h (method 942.05, AOAC 2006). The content of crude fat (EE, method 920.39, AOAC 2006, without HCL hydrolysis), and CF (CF, method 978.10, AOAC 2006) was also analyzed (AOAC., 2006). Gross energy (GE) was determined using a bomb calorimeter (Parr 6300 Calorimeter; Parr Instrument Company, Moline, IL, USA). The level of TiO_2 was determined following the report of Titgemeyer et al., (2001). The Trichloroacetic-acid-soluble protein (TCA-SP) was determined as described by Sriket et al., (2012) and the Agricultural Industry Standard of the People's Republic of China (NY/T 3801-2020). Briefly, 5 g samples were placed into a 250 mL conical bottle, and added 100 mL trichloroacetic acid solution (15%, *w*/*v*) to dissolve and mixed evenly. The solution was filtered with medium speed qualitative filter paper, and the filtrate was transferred to a centrifuge tube after a 5 min standing. Then, the samples were centrifuged at 4000× *g* for 20 min at 4 ℃, and, finally, the CP concentration was determined according to the method of AOAC. The TCA-SP concentration was calculated as the ratio of CP. The concentration of L-lactic acid was determined as that described by Tejero-Sariñena et al., (2012). The amino

acids concentration was determined using an amino acid analyzer (Hitachi L-8800, Tokyo, Japan) after hydrolyzing the samples with 6 M HCl (containing phenol) for 24 h at 110 ℃ in glass tubes sealed under vacuum, and the cysteine and methionine were analyzed as cysteic acid and methionine sulphone by oxidation with performic acid for 16 h at 0 °C and neutralization with hydrobromic acid prior to hydrolysis according to the method, as described by AOAC (method 994.12, (AOAC., 1990). The phytic acid and AFB_1 content of PNM and FPNM samples were measured using a commercial kit (K-PHYT, Megazyme, Wicklow, Ireland) and (Longkefangzhou Bio-Engineering Technology Company, Beijing, China), respectively. Bacterial quantity measurements were conducted using the plate-count method (Laca et al., 2006). After the measurement process, selected colonies were identified using the identification methods described in section 3.5 of Chapter Ⅳ and Section 3.7 of Chapter V. These methods allowed us to determine whether the counted colonies belonged to the species *B. velezensis* or *P. acidilactici*. Three independent samples were measured in triplicate.

3.3 Scanning electron microscopy of PNM and FPNM

The microstructure of PNM and FPNM was analyzed using a field-emission scanning electron microscope (ZEISS Merlin, Oberkochen, Germany), and the samples were gilded in argon by ion sputter coater before observation (Technex, Tokyo, Japan).

3.4 Experiment 1: Determination of ileal digestibility of amino acids

3.4.1 The birds management and sample collection

The experiment was done at the Nankou Experimental Base of the Chinese Academy of Agricultural Sciences. A total of 120 newly hatched Arbor Acres (AA) male broilers (46.3 ± 0.6 g/bird) were purchased from a local commercial hatchery (Beijing Dafa Chia Tai Co. LTD, Beijing, China). During 1–21 d of age, all the birds were fed a corn-soybean meal diet, which were formulated with reference to the nutrient requirements recommended by the feeding standards of China (NY/T 2004) for broilers (1994). At 22 d of age, the birds were weighed after 6 h of fasting, and the birds of similar weight were selected and randomly divided into 3 groups of 6 replicate cages, with 6 chickens per cage (108 in total). The nitrogen-free diet was used to determine the loss of endogenous ileal amino acids in birds (Ullah et al., 2017). The semi-purified diets were formulated with PNM and FPNM based on the nitrogen-free diet, respectively; the PNM and FPNM was used as the only protein source to adjust the CP level. The diets were mixed with 0.4% (*w*/*w*) titanium dioxide (TiO_2) as an indicator. Table 15 showed the ingredient composition and nutrient concentration of the reference and experimental diets. According to the management guidelines of commercial AA broiler, all birds were allowed ad libitum purified water via nipple drinkers and feed in pellet form. During the first week, the room temperature was maintained at 33 ℃ and was gradually decreased, reaching 24 ℃ at 16 days of age. The lighting program was a period of 16 h of light and 8 h of darkness (Aviagen., 2009). On 26 d of age, all birds were euthanized using the intravenous

pentobarbital injection method and immediately dissected, and then ileal digesta was collected in a plastic culture dish. The samples from birds within a replicate cage were pooled together and then immediately stored at −20 ℃ (Wu et al., 2020). The ileal digesta was freeze-dried at −50 ℃, fully ground, and passed through a 0.5 mm screen and stored in airtight containers at −4 ℃ for amino acids analysis.

Table 15. Ingredient composition and nutrient concentration of the experimental diets used in the determination of the standardized ilea amino acid digestibility (dry matter basis)[1]

Component	NFD	Experiment Diet	
		PNM	FPNM
Ingredient composition, %			
Peanut meal	0	42.45	0
Fermented peanut meal	0	0	39.77
Corn starch	72.37	36.84	39.49
Sucrose	15.43	13.00	13.00
Crystallitic cellulose	5.00	0	0
Soybean oil	2.50	3.50	3.50
Limestone	1.27	1.21	1.21
$CaHPO_4$	2.13	1.71	1.73
NaCl	0.30	0.30	0.30
Choline chloride	0.10	0.10	0.10
TiO_2	0.40	0.40	0.40
Mineral premix[2]	0.50	0.50	0.50
Total	100.00	100.00	100.00
Nutrient concentrations[3]			
Metabolic energy (MJ/kg)	12.96	12.84	12.89
Crude protein	0.20	19.98	20.03
Calcium	0.90	0.90	0.90
Available P	0.35	0.35	0.35

(1) Abbreviations: NFD, nitrogen-free diet; PNM, peanut meal; FPNM, fermented peanut meal. (2) The premix provided the following per kilogram diet: vitamin A 10,000 IU, vitamin D_3 2000 IU, vitamin E 10 IU, vitamin K_3 2.5 mg, vitamin B_1 1 mg, vitamin B_2 6 mg, vitamin B_3 10 mg, vitamin B_5 40 mg, vitamin B_6 3 mg, vitamin B_{11} 0.3 mg, vitamin B_{12} 0.01 mg, biotin 0.12 mg, Cu (as copper sulfate) 8 mg, Fe (as ferrous sulfate) 80 mg, Mn (as manganese sulfate) 60 mg, Zn (as zinc sulfate) 40 mg, Se (as sodium selenite) 0.15 mg, I (as potassium iodide) 0.35 mg. (3) Crude protein is the analyzed value (n = 3), and others are the calculated values.

3.4.2. Calculations

The apparent ileal digestibility (AID) values of PNM and FPNM were calculated as followed:

$$AID\ (\%) = 1 - (TiO_2 \text{ in diet}/TiO_2 \text{ in ileal digesta}) \times (AA \text{ in ileal digesta}/AA \text{ in diet}) \times 100\% \quad (1)$$

The endogenous ileal amino acids loss (IAA) values of PNM and FPNM were calculated as follows:

$$IAA\ (mg/kg) = AA \text{ in ileal digesta} \times (TiO_2 \text{ in diet}/TiO_2 \text{ in ileal digesta}) \quad (2)$$

IAA is calculated based on the composition content in the nitrogen-free diet.

The standardized ileal digestibility (SID) values of PNM and FPNM were calculated as follows:

$$SID\ (\%) = AID + (IAA/AA \text{ in diet}) \quad (3)$$

3.5 Experiment 2: Determination of apparent metabolizable energy

3.5.1. The birds management and sample collection

The AME and apparent retention of gross energy (ARGE) were measured used the classical substitution method (Azam et al., 2019). The experiment was also conducted at the Nankou Experimental Base of the Chinese Academy of Agricultural Sciences. A total of 80 newly hatched AA male broilers (45.7 ± 0.5 g/bird) were purchased from a local commercial hatchery. The management procedures and feeding for birds between 1–21 d of age was the same as in Experiment 1. On 22 d of age, the birds were weighed after 6 h of fasting, and birds with similar weight were selected and randomly divided into 3 groups of 6 replicate cages, with 4 chickens per cage (72 in total), and fed 3 experimental diets, which consisted of a corn-soybean meal diet, and the nutrients were formulated to meet the feeding standard of China (NY/T 2004) for broilers (Table 16). The other two diets included test ingredients, PNM or FPNM proportionally replaced 30% of the energy-yielding components of the basal diet. TiO_2 was added to all diets as an indigestible marker. All birds were allowed ad libitum feed and water during the experiment. The dietary adaption period was four days. During the last four days (26 to 30 d), feed intake of each group was monitored, and the excreta samples were collected (spilled feed and feathers were removed) quantitatively daily and pooled within a cage. The collected excreta samples were dried in a forced air drying oven at 65 ℃ for 72 h, fully ground, and passed through a 0.5 mm screen and stored in airtight containers at −4 ℃ for AME and ARGE measurement.

Table 16. Ingredient composition and nutrient concentration of the basal diet used in the determination of the apparent metabolizable energy (dry matter basis).

Component	Basel Diet	PNM Diet	FPNM Diet
Ingredient composition, %			
Corn	56.28	38.62	38.62
Soybean meal, (CP 43%)	30.38	20.85	20.85
PNM	0	30	0
FPNM	0	0	30
Corn gluten meal, (CP 60%)	3.97	2.72	2.72
Soybean oil	4.98	3.42	3.42
$CaHPO_4$	1.73	1.73	1.73
L-Lys	0.06	0.06	0.06
DL-Met	0.12	0.12	0.12
Limestone	1.21	1.21	1.21
NaCl	0.27	0.27	0.27
Choline chloride, (50%)	0.10	0.10	0.10
TiO_2	0.40	0.40	0.40
Mineral premix[1]	0.50	0.50	0.50
Total	100.00	100.00	100.00
Nutrient concentration[2]			
Metabolic energy, MJ/kg	12.96	12.06	12.06
Crude protein, %	20.08	27.85	28.80
Calcium, %	0.90	0.94	0.94
Available P, %	0.35	0.42	0.42
Lys, %	1.00	1.21	1.28
Met + Cys, %	0.76	0.83	0.85

(1) The premix provided the following per kilogram diet: vitamin A 12,000 IU, vitamin D_3 2000 IU, vitamin E 20 IU, vitamin K_3 2.5 mg, vitamin B_1 2 mg, vitamin B_2 6 mg, vitamin B_3 2 mg, vitamin B_5 6 mg, vitamin B_6 6 mg, vitamin B_{11} 0.3 mg, vitamin B_{12} 0.025 mg, nicotinic acid 50 mg, folic acid 1.25 mg, D-pantothenic acid 12 mg, biotin 0.12 mg, Cu (as copper sulfate) 8 mg, Fe (as ferrous sulfate) 80 mg, Mn (as manganese sulfate) 100 mg, Zn (as zinc sulfate) 78 mg, Se (as sodium selenite) 0.15 mg, I (as potassium iodide) 0.35 mg. (2) Crude protein, Lys, Met, and cysteine are the analyzed value (n = 3), and others are the calculated values.

3.5.2. Calculations

The apparent metabolizable energy (AME) values of PNM and FPNM were calculated as follows:

$$IF = TiO_2 \text{ in diet}/TiO_2 \text{ in excreta} \quad (4)$$

$$AME_{diet}\ (MJ/kg) = GE \text{ in diet} - (GE \text{ in excreta} \times IF) \quad (5)$$

$$AME_{PNM \text{ or } FPNM}\ (MJ/kg) = ((AME \text{ of experiment diet} - (AME \text{ of control diet} \times 0.70))/0.30 \quad (6)$$

where IF is the indigestibility factor, and GE is the gross energy.

The apparent retention of gross energy (ARGE) values of PNM and FPNM were calculated as follows:

$$ARGE_{PNM \text{ or } FPNM}\ (\%) = AME \text{ of the ingredient}/GE \text{ of the ingredient} \times 100\% \quad (7)$$

3.6 Statistical analysis

All experimental data were tested for normality by using the Shapiro-Wilk test of normality and Levene's test for homogeneity of variances. All datasets were distributed normally. Afterward, the data were analyzed by a one-factor ANOVA procedure of the SPSS19.0 software package for Windows (SPSS Inc., Chicago, IL, USA), and the indexes were expressed as means with standard error of mean (SEM), where $p < 0.05$ indicated a statistically significant difference.

4. Results

4.1 Effects of solid-sate fermentation on the chemical composition, bacterial quantity and surface microstructure of PNM and FPNM

Table 17. Effects of solid-state fermentation on the chemical composition of PNM and FPNM (dry matter basis, *n*= 3)[1].

Items	PNM	FPNM	SEM	*p*-Value	Changes, %
DM, %	88.65	88.73	0.103	0.723	NS
CP, %	46.85[b]	49.99[a]	0.476	<0.001	6.70
EE, %	1.13	1.11	0.014	0.479	NS
CF, %	6.44[a]	5.48[b]	0.148	<0.001	−14.91
Ash, %	5.60	5.65	0.018	0.108	NS
TCA-SP, %	3.02[b]	19.21[a]	2.441	<0.001	536.09
L-lactic acid, %	0.66[b]	3.56[a]	0.438	<0.001	439.39
Phytic acid, %	1.75[a]	0.59[b]	0.175	<0.001	−66.29
AFB_1, μg/kg	38.37[a]	21.61[b]	2.570	<0.001	−43.68
B. velezensis, CFU/g	-	1.1×10^6	-	-	-
P. acidilactici, CFU/g	-	2.3×10^4	-	-	-

[1] Abbreviations: PNM, peanut meal; FPNM, fermented peanut meal; DM, dry matter; CP, crude protein; EE, crude fat; CF, crude fiber; TCA-SP, Trichloroacetic-acid-soluble protein. Determined in triplicate. "NS" stands for no significant difference. "-" stands for undetected or meaningless. Different letters refer to the significant differences ($p < 0.05$).

The chemical compositions of PNM and FPNM are shown in Table 17. Fermentation of PNM significantly increased the content of CP, TCA-SP and L-lactic acid by 6.70%, 536.09%, and 439.39%, respectively ($p < 0.05$), and decreased the content of CF, phytic acid, and AFB_1 by 14.91%, 66.29%, and 43.68%, respectively ($p < 0.05$). The two inoculated bacteria in the PNM successfully grew and multiplied. Following the final low-temperature drying process, we measured the content of *B. velezensis* in FPNM to be 1.1×10^6 CFU/g, and the content of *P. acidilactici* to be 2.3×10^4 CFU/g.

The physical microstructure of PNM and FPNM were shown in Figure 18. The particles of PNM showed a relatively smooth surface. After fermentation, the large particles were broken down into smaller ones, which tightly pack together in irregular shapes. The surface of FPNM particles exhibits more micropores and cracks compared with that of PNM.

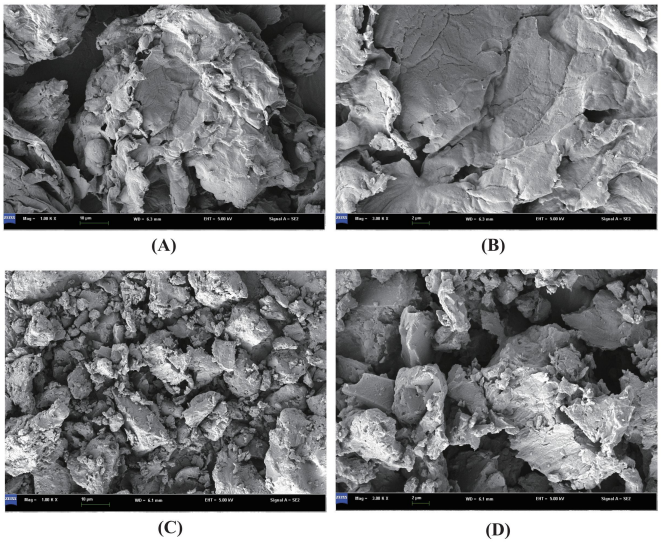

Figure 18. Effects of solid-state fermentation on the surface microstructure of PNM and FPNM. (A,B), scanning electron microscopy images of PNM (1000× & 3000×); (C,D), scanning electron microscopy images of FPNM (1000× & 3000×).

4.2 Effects of solid-state fermentation on the amino acids of PNM and FPNM

The amino acid composition of PNM and FPNM is shown in Table 18. The fermentation of PNM increased the free amino acids level of essential amino

acids, except Arg ($p < 0.05$), and also increased all free amino acids' level of non-essential amino acids ($p < 0.05$). The hydrolyzed amino acids of essential amino acids including Lys, Leu, Val, and Phe were increased after fermentation, while the Arg level was decreased ($p < 0.05$). Fermentation decreased the ratio of Arg to Lys from 3.01 to 1.60. The Gly, Pro, and Ala level of hydrolyzed amino acids of non-essential amino acids was increased after fermentation, while the Ser level was decreased ($p < 0.05$).

Table 18. Effects of solid-state fermentation on the amino acids of PNM and FPNM (dry matter basis, $n = 3$)[1]

Items	Free Amino Acids				Hydrolyzed Amino Acids			
	PNM	FPNM	SEM	*p*-Value	PNM	FPNM	SEM	*p*-Value
Essential amino acids, %								
Met	0.01^{b}	0.22^{a}	0.049	<0.001	0.50	0.59	0.029	0.120
Lys	0.02^{b}	0.59^{a}	0.126	<0.001	1.70^{b}	2.05^{a}	0.080	<0.001
Thr	0.02^{b}	0.19^{a}	0.036	<0.001	1.28	1.33	0.022	0.314
Trp	0 ^{b}	0.01^{a}	0.003	<0.001	0.44	0.48	0.013	0.123
Arg	0.21^{a}	0.03^{b}	0.039	<0.001	5.12^{a}	3.29^{b}	0.392	<0.001
Ile	0.01^{b}	0.39^{a}	0.086	<0.001	1.64	1.73	0.036	0.272
Leu	0.01^{b}	0.94^{a}	0.209	<0.001	3.02^{b}	3.34^{a}	0.089	0.049
Val	0.01^{b}	0.87^{a}	0.191	<0.001	2.00^{b}	2.30^{a}	0.077	0.027
His	0.01^{b}	0.29^{a}	0.062	<0.001	1.12	1.29	0.049	0.088
Phe	0.04^{b}	1.23^{a}	0.267	<0.001	2.45^{b}	2.97^{a}	0.117	0.001
Non-essential amino acids, %								
Gly	0.01^{b}	0.54^{a}	0.118	<0.001	2.70^{b}	3.02^{a}	0.081	0.021
Ser	0.03^{b}	0.08^{a}	0.012	0.019	2.15^{a}	1.85^{b}	0.073	0.008
Pro	0.02^{b}	0.40^{a}	0.085	<0.001	2.13^{b}	2.46^{a}	0.076	0.001
Ala	0.03^{b}	0.95^{a}	0.206	<0.001	1.91^{b}	2.63^{a}	0.162	<0.001
Asp	0.05^{b}	0.67^{a}	0.139	<0.001	5.43	5.69	0.078	0.093
Glu	0.14^{b}	2.01^{a}	0.422	<0.001	8.97	9.48	0.237	0.302
Cys	0^{b}	0.15^{a}	0.034	<0.001	0.55	0.62	0.027	0.171
Tyr	0^{b}	0.45^{a}	0.099	<0.001	1.48	1.46	0.036	0.845
Arg/Lys			—		3.01	1.60	0.308	<0.001

[1] Abbreviations: PNM, peanut meal; FPNM, fermented peanut meal. Different letters refer to the significant differences ($p < 0.05$).

4.3 Ileal digestibility of amino acids of PNM and FPNM

The AID and SID values of amino acids in PNM and FPNM are shown in Table 19. The AID and SID values of essential amino acids (Met, Lys, Leu and Phe) were higher for FPNM than for PNM ($p < 0.05$). Moreover, the AID and SID values of non-essential amino acids of FPNM were both higher than those of PNM, except for Pro ($p < 0.05$).

Table 19. Apparent and standardized ileal digestibility values of amino acids in PNM and FPNM (dry matter basis, $n = 6$)[1]

Items	AID, %				SID, %			
	PNM	FPNM	SEM	*p*-Value	PNM	FPNM	SEM	*p*-Value
Essential amino acids								
Met	85.35^{b}	89.95^{a}	0.830	0.001	88.12^{b}	91.85^{a}	0.722	0.003
Lys	81.87^{b}	85.72^{a}	0.701	0.001	83.70^{b}	87.32^{a}	0.672	0.001
Thr	78.63	80.76	0.678	0.119	84.74	86.31	0.642	0.239
Trp	83.48	86.37	0.749	0.058	86.44	88.91	0.716	0.082
Arg	90.97	91.40	0.334	0.542	91.68	91.74	0.328	0.917
Ile	84.27	86.73	0.638	0.053	86.38	88.57	0.616	0.071
Leu	85.80^{b}	88.27^{a}	0.536	0.012	87.71^{b}	89.84^{a}	0.519	0.032
Val	84.44	86.72	0.668	0.087	87.32	89.28	0.644	0.134
His	87.43	89.30	0.463	0.056	89.03	90.65	0.442	0.061
Phe	88.53^{b}	90.96^{a}	0.470	0.003	90.14^{b}	92.36^{a}	0.446	0.005
Non-essential amino acids								
Gly	67.45^{b}	72.40^{a}	0.965	0.003	69.51^{b}	74.12^{a}	0.925	0.005
Ser	81.01^{b}	83.88^{a}	0.675	0.025	84.25^{b}	87.10^{a}	0.664	0.023
Pro	84.44	86.43	0.523	0.051	87.36	88.99	0.494	0.100
Ala	83.51^{b}	86.80^{a}	0.634	0.003	85.80^{b}	88.80^{a}	0.601	0.005
Asp	83.62^{b}	86.44^{a}	0.654	0.022	85.27^{b}	87.81^{a}	0.628	0.035
Glu	87.83^{b}	89.82^{a}	0.411	0.007	88.95^{b}	90.83^{a}	0.407	0.012
Cys	70.71^{b}	79.60^{a}	1.368	<0.001	79.52^{b}	85.77^{a}	0.984	<0.001
Tyr	88.31^{b}	91.29^{a}	0.632	0.009	90.77^{b}	93.35^{a}	0.589	0.019

[1] Abbreviations: PNM, peanut meal; FPNM, fermented peanut meal; AID, apparent ileal digestibility; SID, standardized ileal digestibility. Different letters refer to the significant differences ($p < 0.05$).

4.4 Apparent metabolizable energy of PNM and FPNM

Table 20. Apparent metabolizable energy and apparent retention of gross energy in PNM and FPNM (dry matter basis, *n*= 6)[1]

Items	PNM	FPNM	SEM	*p*-Value
AME, MJ/kg	10.68	10.89	0.152	0.508
ARGE, %	57.70	58.51	0.810	0.640

[1] Abbreviations: PNM, peanut meal; FPNM, fermented peanut meal; AME, apparent metabolizable energy; ARGE, apparent retention of gross energy.

The AME and ARGE values of PNM and FPNM are shown in Table 20. There was no significant difference in AME or ARGE between PNM and FPNM ($p > 0.05$).

5. Discussion

China is currently the largest producer, consumer, and exporter of peanuts in the world. According to the report of the Food and Agriculture Organization of the United Nations of 2020, the annual peanut production was about 18 million tons in China, of which more than 50% (9 million tons) was used for crushing, which produced about 4.5–5.9 million tons of PNM (Zhao et al., 2012; Fletcher and Shi, 2016). In early studies, some negative effects of PNM were found, which limit its application. Costa et al., (2001b) found that, due to the imbalance of amino acids, broilers fed on corn-peanut meal diets required additional supplementation of Lys, Thr, and Met to achieve the same growth performance as corn-soybean meal diets. Xia et al., (2022b) also found that replacing soybean meal with peanut meal in the diet reduced the egg production and antioxidant capacity of egg ducks.

Solid-state fermentation has been studied and applied in the food and feed industry for a long time. Its application can improve the nutritional value of the substrate, as well as produce organic acids, enzymes, and other beneficial metabolites in the body (Wang et al., 2017a; Olukomaiya et al., 2019). The present study found that fermentation changed the chemical composition of PNM. Yang et al., (2016a) reported that the CP content increased by 6.96% after fermentation with *Bacillus licheniformis*, which is close to 6.70% in this study. However, this was not an actual increase in protein content since no additional nitrogen sources were supplemented during fermentation. Hu et al., (2016) considered that the increase in CP content was mainly associated with a decrease in the concentration of non-structural carbohydrates, and the proportion of CP that was compensated for increased due to a decrease in dry matter content. Additionally, except for proteases, strains of *Bacillus* spp. produce fiber-degrading enzymes, such as cellulases, hemicellulases, and β-glucanases (Wongwilaiwalin et al., 2010). The decrease in CF content may be related to the fiber-degrading enzymes secreted by the bacteria used for fermentation.

TCA-SP is a parameter that indicates the degree of protein decomposition, mainly including small peptides with molecular weight less than 10 kDa; free amino acids; and small amounts of non-protein nitrogen compounds (Gao et al., 2022a). Yang et al., (2016b) reported that the content of TCA-SP in fermented PNM was increased by 400.37%, which was consistent with our findings. The positive effect of fermentation on protein of PNM facilitated protein utilization, and amino acids in the form of small molecular peptides were more easily transported by intestinal epithelial cells than their free form. The increase of L-latic acid content can lower the pH value of raw materials and resist the proliferation of mold and harmful microorganisms, which can prolong the storage time of the product and solve the problem that PNM is not easy to store (Abdollahi et al., 2020). PNM contains many anti-nutritional factors such as phytic acid, trypsin inhibitor and plant hemagglutinin, among which phytic acid is an important factor for inhibiting the utilization of nutrients in PNM. *Bacillus subtilis*, *Bacillus cereus*, and other *Bacillus* spp. can secrete phytase, which can hydrolyze phytic acid into inositol and phytate, and release nutrients such as amino acids and mineral elements combined with phytic acid in the process of enzymatic hydrolyzation, thus improving the utilization rate of nutrients in raw materials (Shobirin et al., 2010; Ahmad et al., 2017). Our study found that the level of phytic acid in PNM was significantly reduced after the solid-state fermentation, which may be related to the production of phytase by microorganism. The study of Castro-Alba et al., (2019) showed that the fermentation of pseudocereal flours by *Lactobacillus* degraded phytic acid and thus improved the utilization of mineral elements. Concomitantly, researchers also reported that phytase produced by microorganisms was selective for the degradation of phytic acid, and not all raw materials can achieve the same degradation efficiency, which suggested that we need to use effective microorganisms for fermentation. In this study, the degradation of phytic acid by microorganisms was not only due to the phytase, but also because the L-lactic acid produced by the LC-9-1 reduced the pH value of PNM, and the change in pH value can provide conditions for the activation of microbial phytase and endogenous plant phytase (Coulibaly et al., 2010). AFB_1 is the most carcinogenic of all natural toxins, and with a detection rate of nearly 100% in PNM (Kana et al., 2013). Through our experiments, we found that fermentation of PNM by LB-Y-1 and LC-9-1 significantly reduced the level of AFB_1 in PNM and improved the quality of PNM. Probiotics generally clear AFB_1 by inhibiting spore formation of *Aspergillus flavus* and *Aspergillus parasiticus*, which produces AFB_1, or by enzymatic cleavage of the lactone ring of AFB_1 (Alberts et al., 2009; Xu et al., 2013). For this study, both effects may be present simultaneously. Wang et al., (2018a) have reported that *B. velezensis* has great potential as a feed additive to remove mycotoxins in animal feed. What's more, the fermentation strain LC-9-1 used in this experiment has the function of inhibiting the proliferation of some pathogenic microorganisms (Li et al., 2023b).

In this study, fermentation significantly increased the concentration of free amino acids in PNM, excluding Arg. The increase is based on the activity of peptidases, and is highly dependent on the microorganisms involved in

fermentation (Shukla et al., 2020). However, we also found a decrease in the concentration of Ser and Arg as hydrolyzed amino acids, which may be related to the utilization of microorganisms. Similar to our results, in a study on solid-state fermentation of soybean meal, Ser and Arg decreased by 22.04% and 21.73%, respectively (Li et al., 2022b). Compared to fish meal (0.76) and soybean meal (1.15), the ratio of Arg to Lys in PNM is 3.01, which is seriously imbalanced (Jia et al., 2022). Excessive Arg will lead to the antagonism of amino acids in animals and inhibit the absorption of Lys, and affect the normal growth and development of animals. We found that the Lys content of hydrolyzed amino acids in the FPNM was significantly increased by probiotics fermentation and the ratio of Arg to Lys was reduced to 1.6, making the amino acid composition of FPNM closer to the ideal amino acid composition.

The endogenous amino acid losses in broilers were corrected by the classical NFD method, and the result was similar to that in the report of Barua et al., (2021), which proved that the method was reasonable. Our experiment showed that the proportion of main endogenous amino acids such as Glu, Asp, Thr, and Ser in ileum increased in broilers fed with FPNM (Ravindran, 2021). There are limited studies on the effect of FPNM on ileal amino acid digestibility in broiler chickens, but we have found in other studies on plant-based protein feedstuffs that rapeseed meal and soybean meal fermented by probiotics increase the AID and SID of most amino acids in broiler chickens, which is similar to our research in which the AID and SID of partial essential and non-essential amino acid were both increased in broilers fed FPNM (Wu et al., 2020; Li et al., 2022b). Additionally, it was found that the digestibility of dry matter of PNM in vitro was increased by 10.18% after fermentation by *Bacillus licheniformis* (Yang et al., 2016b). The reason for the increase in amino acid digestibility may be related to the improvement of protein molecules in PNM after fermentation; more peptides and free amino acids were absorbed by the animal intestine, and the peptides also promoted the utilization of amino acids by the animal intestine (Gilbert et al., 2008b). Furthermore, the fermentation by probiotics reduced the interference of CF on the absorption of nutrients in PNM; specific manifestations include the decreased viscosity of digesta and better binding between digestive enzymes and substrates. These also promoted the digestibility of amino acids (Upadhaya and Kim, 2015). Additionally, the decrease in the phytic acid level can also promote the digestion of amino acids. It's well known that phytic acid combines with endogenous proteases (such as pepsin and trypsin) to form complexes that reduce amino acids digestibility, especially the AID of Cys, Asp acid and Gly, ultimately leading to lower feed conversion rates (Walk and Olukosi, 2019; Walk and Rama Rao, 2020a, 2020b). As the limiting amino acids in livestock diets, the improvement in the digestibility of Met and Lys can promote amino acid balance and utilization efficiency in diets (Farkhoy et al., 2012). Moreover, amino acids balance can accurately predict the nutritional value of feedstuff and reduce the impact of unnecessary nitrogen emissions on environmental pollution, and also provide a new theory and method for the study of low protein diets. In this study, we

obtained an AME of 10.68 MJ/kg for PNM, which was close to the value of 10.88 MJ/kg published in the Chinese Feed Composition and Nutritional Value (2019 version) (CFIC., 2019). However, no significant difference was derived between PNM and FPNM for AME and ARGE. Similar to our results, Li et al., (2022b) did not find a significant difference in AME between soybean meal and fermented soybean meal in broilers. The availability of energy in PNM and FPNM mainly depends on the balance of energy-yielding components in the feed and the factors that inhibit its utilization. Reduction in CF and anti-nutritional factors would favor the utilization of nutrients, and degraded CF was converted into disaccharides and eventually into glucose, which was more readily utilized by the body (Horn et al., 2012; Jaworski et al., 2015; Zhou et al., 2016). However, fermentation itself is an energy-consuming process, in which the microorganisms consume carbon sources such as monosaccharides, disaccharides, oligosaccharides, starch, and cellulose from the substrate for proliferation and biotransformation (Shi et al., 2021). Thus, the energy that can be utilized by the body may not increase, which we believe has led to the absence of any difference in the AME of PNM and FPNM.

6. Conclusions

Solid-state fermentation significantly improved the quality of PNM by increasing the concentration of CP, TCA-soluble protein, and L-lactic acid, decreasing the concentration of CF, phytic acid, and AFB_1; and fermentation improved the composition of amino acids including free and hydrolyzed amino acids. Our study also demonstrated that fermentation improved the AID and SID of most amino acids in PNM but did not affect the AME or ARGE. The present study provides basic data and theory for the study of FPNM and provides references for the design of broiler formulations.

Chapter VII

Dietary fermented peanut meal supplementation alters growth performance, meat quality and oxidative stability in broilers

Based on previous research findings, it has been demonstrated that fermentation can enhance the digestibility of ileal amino acids in peanut meal for broilers. However, there is limited information regarding the impacts of fermented peanut meal on broiler growth performance and meat quality. Therefore, the objective of this study is to assess the effects of incorporating different proportions (5%, 10%, and 15%) of PNM and FPNM into the broiler diet on parameters such as growth performance, antioxidant capacity, meat quality, and oxidative stability.

Running title: The application of fermented peanut meal in broilers production
Key words: fermented peanut meal, broiler, growth performance, meat quality, oxidative stability

Chapter VII. Dietary fermented peanut meal supplementation alters growth performance, meat quality and oxidative stability in broilers

1. Abstract

Fermented peanut meal (FPNM) is a product of solid-state fermentation technology, which is transformed through microbial fermentation using peanut meal (PNM) as the main substrate. In this study, the diets containing FPNM were compared to the diets containing corn-soybean meal and PNM on the growth performance, meat quality, and oxidative stability of broiler chickens. To address this, a total of 420 Arbor Acres (AA) 1-day-old, male broiler broilers were randomly allocated into 7 treatment groups, with 6 replicates, containing 10 broilers in each replicate. The treatment groups were control group (CON) fed a corn-soybean based diet; PNM and FPNM treatment group fed the basal diet supplemented with 5%, 10% and 15% PNM and FPNM. Over the 42 days experiment, broilers fed diets containing 10% FPNM exhibited significantly ($p < 0.05$) higher body weights (21 and 42 d of age) and average daily gain (overall phase), compared to the PNM groups. Correspondingly, the 10% FPNM group exhibited the lowest feed conversion ratio (feed intake : weight gain) during the grower period (22-42 days) and overall when compared to all other treatments. In terms of meat quality, the breast muscle of broilers in the 10% FPNM group exhibited higher meat color (redness, a*), $pH_{24\ hour}$ values, and oxidative stability, compared to the 10% PNM group. The supplementation of 10% PNM resulted in decreased levels of Lys, Met, and Thr in the breast muscle, compared to the CON group ($p < 0.05$), while the addition of 10% FPNM mitigated these changes. Furthermore, the 10% FPNM group exhibited enhanced antioxidant capacity, as evidenced by higher levels of T-AOC and SOD, and lower levels of MDA, compared to the 10% PNM group. In conclusion, FPNM has a positive impact on broiler growth performance, meat quality, and oxidative stability. Overall considering all performances, the optimal performance can be obtained when the supplementation amount is 10%.

2. Introduction

Chicken is gaining increasing acceptance among consumers worldwide due to its significant price advantages compared to other types of meat. Moreover, its low fat content and cholesterol levels, which are highly appreciated by consumers (Fan et al., 2018). However, the meat quality has certain limitations that require improvement. One area that needs enhancement is the meat color, as the breast muscle can sometimes appear pale, which may not meet the visual expectations of consumers who prefer a vibrant and appealing appearance (Fletcher et al., 2000). Another aspect that could be improved is the flavor profile of the chicken (Wang

et al., 2017b). While it is generally mild, some consumers may desire a more robust and flavorful taste. Additionally, addressing the issue of oxidation is indeed an important aspect to consider in improving chicken. Oxidation can lead to undesirable changes in the color, flavor, and overall quality of the meat (Yang et al., 2020a). Focusing on enhancing the color, flavor, and oxidative stability of chicken breast muscle can further elevate its desirability and meet the evolving preferences of consumers.

Fermented products have been widely reported to improve the growth performance and meat quality of broiler chickens. Research conducted by Li et al., (2020) investigated the impact of fermented soybean meal on broiler growth performance. The results demonstrated that broilers fed with 5% fermented soybean meal exhibited reduced the ratio of feed conversion ratios (feed intake : weight gain, FCR), increased average daily gain (ADG), and enhanced immune responses compared to those fed with non-fermented soybean meal . Furthermore, a study by Zengin et al., (2022) evaluated the effects of fermented distillers grains on broiler meat quality. The researchers found that broilers fed with fermented distillers grains had a positive effect on FCR, darker meat color and improved antioxidant status of the breast meat compared to those fed with corn-soybean meal . Peanut meal (PNM) is a byproduct obtained from peanuts after the extraction of oil through high-temperature pressing. It is commonly used as a feed ingredient due to its availability and relatively low cost. However, several factors limit peanut meal from being regarded as a high-quality feed ingredient, including an imbalance in amino acid composition, the presence of anti-nutritional factors such as phytic acid, its susceptibility to aflatoxin contamination, and the potential presence of pathogenic microorganisms (Batal et al., 2005; Tola and Kebede, 2016). Proper processing, quality control, and supplementation strategies are essential to mitigate these limitations and maximize the utilization of peanut meal in animal feed formulations.

Previously, we have demonstrated that fermentation can improve the digestibility of ileal amino acids in PNM for broilers (Li et al., 2023c). However, limited information is available regarding the effects of FPNM on broiler growth performance and meat quality. The objective of this study is to evaluate the effects of dietary addition of different proportions (5%, 10% and 15%) of PNM and FPNM on broiler growth performance, antioxidant capacity, meat quality and oxidative stability

3. Materials and methods

3.1 Preparation of FPNM

The preparation of FPNM involved the conventional solid-state fermentation method, utilizing *Bacillus velezensis* LB-Y-1 (3.0×10^8 CFU/mL) and *Pediococcus acidilactici* LC-9-1 (1.0×10^8 CFU/mL) as the starter culture. The specific procedures followed are described in section 3.1 of Chapter VI.

3.2 Experimental design, bird management and sample collection

The experiment was conducted in Nankou pilot base of the Chinese Academy of Agricultural Sciences. The proceedings of this research were licensed by the ethical approval of the Animal Care and Use Committee of the Institute of Feed Research of the Chinese Academy of Agricultural Sciences (Statement No. AEC-CAAS-20191106, Beijing, China).

A total of 420 newly hatched Arbor Acres (AA) male broilers (38.2±0.5 g/bird, equal numbers of male and female) were purchased from a local commercial hatchery (Beijing Dafa Chia Tai Co. LTD, Beijing, China). The broilers were randomly assigned to 7 treatments, each treatment comprised 6 replicates with 10 birds each. The experiment lasted for 42 days with two feeding phases, starter (1-21 d) and grower (22-42 d). The control group (CON) was fed the corn-soybean meal basal diet, and formulated to meet the nutritional requirements of the birds as determined by the National Research Council (1994) and Chinese Ministry of Agriculture (2004) (National Research Council, 1994; Ministry of Agriculture of the People's Republic of China, 2004). The groups 2-4 were fed with 5%, 10%, and 15% PNM, respectively. The groups 5-7 were fed with 5%, 10%, and 15% FPNM, respectively. The composition and nutritional levels of the diets are shown in Table 21 and Table 22.

Table 21. Ingredient and nutrient composition of basal broiler diets (1-21 d)

Items	CON	Group PNM			Group FPNM		
		PNM5	PNM10	PNM15	FPNM5	FPNM10	FPNM15
Ingredient (%)							
Corn	56.17	56.46	56.13	56.06	57.32	57.17	56.46
Soybean meal (43% CP)	31.58	26.37	21.68	16.91	25.69	20.97	16.85
Corn gluten meal (61% CP)	4.90	4.80	4.80	4.62	4.70	4.63	4.50
PNM	0	5.00	10.00	15.00	0	0	0
FPNM	0	0	0	0	5.00	10.00	15.00
Soybean oil	2.99	2.91	2.86	2.80	2.83	2.70	2.65
$CaHPO_4$	1.99	1.99	1.99	2.00	1.99	2.00	2.00
DL-Met	0.14	0.15	0.16	0.17	0.15	0.16	0.17
L-Lys	0.16	0.23	0.28	0.33	0.23	0.26	0.27
Limestone	1.33	1.34	1.34	1.35	1.34	1.35	1.34

NaCl	0.27	0.28	0.28	0.28	0.28	0.28	0.28
Choline chloride	0.22	0.22	0.23	0.23	0.22	0.23	0.23
Vitamin and mineral premix[1)]	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Total	100	100	100	100	100	100	100
Nutrient concentrations[2)]							
Metabolic energy (MJ/kg)	12.54	12.54	12.54	12.54	12.54	12.54	12.54
Crude protein	21.23	21.16	21.45	21.46	21.31	21.51	21.38
Calcium	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Available P	0.45	0.45	0.45	0.45	0.45	0.45	0.45
Lys	1.15	1.15	1.15	1.15	1.15	1.15	1.15
Met	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Met + Cys	0.85	0.84	0.83	0.83	0.84	0.84	0.83
Arg	1.31	1.40	1.51	1.61	1.29	1.30	1.33

[1)] The premix provided the following per kilogram diet: vitamin A 10, 000 IU, vitamin D_3 2000 IU, vitamin E 10 IU, vitamin K_3 2.5 mg, vitamin B_1 1 mg, vitamin B_2 6 mg, vitamin B_3 10 mg, vitamin B_5 40 mg, vitamin B_6 3 mg, vitamin B_{11} 0.3 mg, vitamin B_{12} 0.01 mg, biotin 0.12 mg, Cu (as copper sulfate) 8 mg, Fe (as ferrous sulfate) 80 mg, Mn (as manganese sulfate) 60 mg, Zn (as zinc sulfate) 40 mg, Se (as sodium selenite) 0.15 mg, I (as potassium iodide) 0.35 mg. [2)]Prepared as 4 g of titanium dioxide mixed with 20 g of ground corn. [3)] Calculated nutrient concentrations.

Table 22. Ingredient and nutrient composition of basal broiler diets (22-42 d)

Items	CON	Group PNM			Group FPNM		
		PNM5	PNM10	PNM15	FPNM5	FPNM10	FPNM15
Ingredient (%)							
Corn	58.95	59.41	59.02	58.93	59.28	59.58	59.82
Soybean meal (43% CP)	28.69	23.11	18.60	13.84	23.28	18.32	13.07
Corn gluten	4.50	4.36	4.24	4.11	4.39	4.15	4.00

meal (61% CP)							
PNM	0	5.00	10.00	15.00	0	0	0
FPNM	0	0	0	0	5.00	10.00	15.00
Soybean oil	4.00	4.13	4.07	3.97	4.08	3.91	4.00
$CaHPO_4$	1.69	1.70	1.70	1.70	1.70	1.70	1.70
DL-Met	0.04	0.07	0.08	0.09	0.07	0.08	0.09
L-Lys	0.06	0.14	0.19	0.25	0.12	0.16	0.21
Limestone	1.25	1.25	1.26	1.27	1.25	1.26	1.27
NaCl	0.27	0.28	0.28	0.28	0.28	0.28	0.28
Choline chloride	0.30	0.30	0.31	0.31	0.30	0.31	0.31
Vitamin and mineral premix[1)]	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Total	100	100	100	100	100	100	100
Nutrient concentrations[2)]							
Metabolic energy (MJ/kg)	12.96	12.96	12.96	12.96	12.96	12.96	12.96
Crude protein	20.05	19.90	20.18	20.37	20.14	20.36	20.41
Calcium	0.90	0.90	0.90	0.90	0.90	0.90	0.90
Available P	0.40	0.40	0.40	0.40	0.40	0.40	0.40
Lys	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Met	0.40	0.40	0.40	0.40	0.40	0.40	0.40
Met + Cys	0.71	0.72	0.71	0.71	0.72	0.72	0.71
Arg	1.22	1.30	1.41	1.51	1.21	1.20	1.22

[1)] The premix provided the following per kilogram diet: vitamin A 10, 000 IU, vitamin D_3 2000 IU, vitamin E 10 IU, vitamin K_3 2.5 mg, vitamin B_1 1 mg, vitamin B_2 6 mg, vitamin B_3 10 mg, vitamin B_5 40 mg, vitamin B_6 3 mg, vitamin B_{11} 0.3 mg, vitamin B_{12} 0.01 mg, biotin 0.12 mg, Cu (as copper sulfate) 8 mg, Fe (as ferrous sulfate) 80 mg, Mn (as manganese sulfate) 60 mg, Zn (as zinc sulfate) 40 mg, Se (as sodium selenite) 0.15 mg, I

(as potassium iodide) 0.35 mg. [2)]Prepared as 4 g of titanium dioxide mixed with 20 g of ground corn. [3)]Calculated nutrient concentrations.

The broilers were housed in an environmentally controlled facility (fiberglass feeders and plastic net floor). The temperature was controlled at 33-35 ℃ during the first week. During the second week, and it was lowered by 1-2 ℃ per/d thereafter, but not lower than 24℃. The broilers were allowed free access to feed and water, with 16 h light: 8 h dark cycle. Relative humidity was controlled at 60 to 70% during days 1-7, and then at 50 to 60% for the remainder of the experiment. The vaccination and disinfection were carried out in accordance with the AA Broiler Guidelines for Feeding and Managements, and the cage was cleaned twice a day at 08:00 and 17:00 (Aviagen., 2009). Throughout the experiment, any broilers that became sick, injured, or died were immediately removed and weighed separately.

At 1, 21, and 42 d of age, the broilers were weighed on a replicate basis after fasting for 8 hours, The feed was weighed weekly for each replication to calculate the body weight (BW), average daily gain (ADG), average daily feed intake (ADFI), and feed conversion ratio (feed/gain, F/G) of the broilers. At 42 d of age, one broiler close to average BW of the cage was selected and weighed. The blood samples were collected from the wing vein and transfer to anticoagulant-free vacuum test tube. Centrifuge at 3,000×g for 10 minutes at 4 ℃ to obtain serum, which is then stored at -20 ℃ for measuring antioxidant activity. After blood sampling, the broilers were slaughtered through the intravenous injection of pentobarbital sodium and jugular exsanguination. The carcass traits was measured based on the previous method (Abo Ghanima et al., 2020). The weight of the abdominal fat was expressed as the percentage of dressed weight, and the weight of the breast muscle and tight muscle were expressed as the percentage of eviscerated weight. The left breast muscle samples were collected to evaluate the meat quality. The right breast muscle samples were collected for chemical composition and amino acid analysis. All samples were vacuum packaged individually and stored immediately at -20 ℃ until analysis.

3.3 Meat quality evaluation

Following the method of Savaris et al., (2021), the meat color including lightness (L*), redness (a*) and yellowness (b*) was measured using a colorimeter (CR 400, Minolta, Osaka, Japan) at 45 minutes after slaughter. Each sample was measured from 3 locations, and the average values were taken as the final result.

The pH value of the breast muscles was measured using a portable pH meter (Sartorius PB-10, Sartorius, Göttingen, Germany) at 45 minutes and 24 hours after slaughter. Three different spots were chosen on each sample, and the results were averaged.

The determination of drip loss and cooking loss were performed according to the protocol described by Gao et al., (2022b). The drip loss were measured at 45 min after the birds were slaughtered. Approximately 10 grams of breast muscle

were weighed (W1) and placed in a polyethylene bag. The meat was suspended vertically by attaching one end with a thin thread, and the bag was filled with nitrogen to create expansion and tightly sealed. Subsequently, the meat was hung in a controlled environment at 4 ℃ for 24 hours. The surface moisture was then absorbed using filter paper, and the meat was reweighed (W2). The drip loss was calculated using the formula (W1-W2)/W1×100%. For the assessment of cooking loss, another approximately 30-gram piece of breast muscle was weighed, placed in a polyethylene bag, and vacuum-sealed. Subsequently, the meat was heated in a water bath at 85 ℃ until the internal temperature reached 77℃. After reaching the desired temperature, it was then cooled to room temperature under running water. The surface moisture was absorbed with filter paper and weighed again (W2). The cooking loss was calculated using the formula (W1-W2)/W1×100%. The determination of shear force was performed using a shear force device equipped with Warner-Bratzler shear blades (Texture Analyzer, Baosheng Industrial Development Co., Ltd, Shanghai, China), following the manufacturer's instructions.

The chemical composition of breast muscle was determined according to the Association of Analytical Chemists method (AOAC International, 2000). Specifically, the moisture content was tested by the oven-drying method (110℃, 24h). The crude protein (CP) content was determined by the Kjeldahl method (N×6.25). The crude fat content was determined following the Soxhelt extraction method. The ash content was determined via the dry ashing method (550℃, 4h).

The oxidative stability of the breast muscle samples was determined following the previously described method (Bai et al., 2016). Briefly, the samples were homogenized on ice with 0.9% NaCl solution in a weight-to-volume ratio of 1:9. After centrifugation at 4,000×g for 10 minutes (4℃), the supernatant was collected for subsequent measurements. Commercial kits (BC4755 and BC4770, Solarbio, Beijing, China) were used to determine the scavenging activity of 1,1-diphenyl-2-pierylhydrazy (DPPH) radicals and 2,2'-Azinobis-(3-ethylbenzthiazoline-6-sulphonate) (ABTS) radicals. All procedures were performed according to the manufacturers' instructions. It is noteworthy that the radical scavenging activity was normalized by the protein content of the samples (mg/ml). The protein concentration of supernatant was measured using a protein assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The calculation formula is as follows:

$$\text{Radical scavenging activity} = [\ (A_{control} - A_{tissue})\ /\ (A_{control} \times \text{protein content})\ ] \times 100\%$$

Where: $A_{control}$ was the absorbance of the control, and A_{tissue} referred to the absorbance of the tissue supernatant.

Based on the method reported by (Wang et al., 2014), inosinic acid (inosine 5'-monophosphate, IMP) was determined in the breast muscle using high-performance liquid chromatography (HPLC). The HPLC was performed on an Agilent 1200 Series System (Agilent, Waldbronn, Germany) equipped with a TC-C18 chromatographic column (5 μm, 4.6 mm×250 mm). The mobile phase

consisted of a mixture of 8% methanol and 92% phosphoric acid solution of 0.5% concentration (V/V). The flow rate was 1.0 mL/min, and the UV detection wavelength was set at 254 nm.

3.4 Amino acid profile of the meat

The amino acid content in the breast muscle was determined according to the method described by Su et al., (2022). Approximately 60 mg of breast muscle sample was placed in a 1.5 mL centrifuge tube and homogenized with 1.0 mL of extraction solution for 240 s, followed by ultrasonication in an ice-water bath for 30 min. After standing at -40 ℃ for 50 min, the samples were centrifuged at 12,000×*g* for 15 min. The supernatant was then freeze-dried and used for analysis. The amino acid analysis was performed using an amino acid analyzer (Hitachi L-8800, Tokyo, Japan), and the specific methods are described in section 3.2 of Chapter VI.

3.5 Serum antioxidant performance

According to the manufacturer's instructions, commercial kits (A015-2-1, A001-3-2, A007-1-1, A005-1-2 and A003-1-2, Nanjing Jiancheng Bioengineering Institute, Nanjing, China) were used to determine the total antioxidant capacity (T-AOC), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-PX), and the concentration of malondialdehyde (MDA) in the serum.

3.6 Statistical analysis

The data were analyzed using the SPSS 19.0 software package for Windows (SPSS Inc., Chicago, IL, USA), and the results were expressed as means with standard error of mean (SEM). Prior to analysis, a test for homogeneity of variances was conducted using ANOVA. Polynomial contrasts were used to test linear and quadratic responses to the increasing levels of PNM or FPNM in the diets. Significant differences between groups were determined using Duncan's multiple range test. A *p*-value less than 0.05 was considered statistically significant.

4. Results

4.1 Growth performance and carcass traits

The birds grew normally and performed well, the mortality rate was 1.8% during the total experimental period, that was not related to the dietary treatments (data not shown). The response of growth performance for the starter (0 to 21 d of age), grower (22 to 42 d of age), and overall (0 to 42 d of age) phases are presented in Table 23. Compared with the CON group, the PNM treatment groups (5%, 10%, 15%) showed a linear decrease in body weight (BW) and average daily gain (ADG) with increasing supplementation levels. Additionally, there was a tendency of increased feed conversion ratio (FCR). The ADG of the 15% PNM group was significantly lower than the CON group in the starter, grower phase and overall ($p < 0.05$). The FCR of the 15% PNM group was significantly higher than the CON group during both the grower phase and overall ($p < 0.05$). As for the FPNM treatment groups, there were no significant differences observed in BW, ADG, or average daily feed intake (ADFI) when compared to the CON group, but

the FCR of the 10% FPNM group was significantly lower than the CON group during the overall phase ($p < 0.05$). In addition, the BW of the FPNM treatment groups increased quadratically with increasing FPNM supplementation level, reaching a maximum at 10% supplementation. Correspondingly, the FCR of the 10% FPNM group was the lowest among the three groups. The carcass traits of the birds at 42 d of age showed that there were no significant differences in carcass traits, including dressing yield, eviscerated yield, breast muscle yield, thigh muscle yield, and abdominal fat yield among all groups. It is noteworthy that, as the level of FPNM supplementation increased, there was a linear decrease in abdominal fat yield. In conclusion, the supplementation of PNM at level of 15% has a negative impact on the growth performance of broilers. However, the supplementation of FPNM at levels ranging from 5% to 15% does not have a detrimental effect on growth performance, and at a 10% supplementation level, it improves the FCR compared to the control group.

Table 23. Effect of dietary PNM and FPNM supplementation on growth performance and carcass traits in broilers (*n*=6)

Items	Days	CON[1]	Supplemental PNM[2] level (%)			Supplemental FPNM[3] level (%)			SEM	*p*-value	Effect of PNM[2]		Effect of FPNM[3]	
			5	10	15	5	10	15			L	Q	L	Q
BW, g	0	38.7	38.0	37.8	38.1	38.2	38.2	37.9	0.105	0.454	NS	NS	NS	NS
	21	780^{ab}	777^{ab}	756^{bc}	727^{c}	799^{a}	809^{a}	776^{ab}	6.137	0.003	0.006	0.016	NS	NS
	42	2569^{abc}	2542^{bc}	2490^{cd}	2418^{d}	2602^{ab}	2647^{a}	2527^{bc}	15.709	0.001	0.004	0.014	NS	0.041
ADG, g/(bird-d)	1-21	34.98^{ab}	34.86^{ab}	34.12^{bc}	32.80^{c}	36.25^{a}	36.71^{a}	35.12^{ab}	0.297	0.004	0.020	0.047	NS	NS
	22-42	84.08^{a}	83.98^{a}	81.94^{ab}	79.68^{b}	84.25^{a}	83.74^{a}	81.08^{ab}	0.478	0.045	0.005	0.014	NS	NS
	1-42	60.25^{abc}	59.63^{bc}	58.38^{cd}	56.67^{d}	61.05^{ab}	62.11^{a}	59.25^{bc}	0.373	0.001	0.004	0.014	NS	0.041
ADFI, g/(bird-d)	1-21	47.14	46.68	47.27	45.90	47.73	47.68	45.70	0.236	0.086	NS	NS	NS	0.024
	22-42	141.88	141.15	138.48	137.96	141.39	138.82	135.50	0.668	0.107	NS	NS	0.005	0.014
	1-42	94.51^{ab}	93.92^{ab}	92.88^{abc}	91.93^{bc}	94.73^{a}	93.26^{abc}	90.60^{c}	0.369	0.021	0.025	NS	0.002	0.002
FCR, g/g	1-21	1.34^{abc}	1.35^{abc}	1.39^{ab}	1.40^{a}	1.33^{bc}	1.30c	1.30^{c}	0.009	0.008	0.049	NS	NS	NS
	22-42	1.69^{b}	1.69^{b}	1.69^{b}	1.73^{a}	1.68^{b}	1.66^{b}	1.67^{b}	0.006	0.023	0.024	NS	NS	NS
	1-42	1.57^{bc}	1.58^{abc}	1.59^{ab}	1.62^{a}	1.55^{bc}	1.50^{d}	1.53^{cd}	0.008	0.001	0.037	NS	0.011	0.009
Dressing yield, %	42	88.88	87.98	91.00	89.15	90.03	90.99	91.12	0.360	0.105	NS	NS	NS	NS
Eviscerated	42	74.81	74.46	74.53	73.43	73.88	75.34	75.85	0.310	0.378	NS	NS	NS	NS

yield, %													
Breast muscle yield, %	42	26.89	27.47	27.60	26.29	27.22	27.13	28.77	0.279	0.397	NS	NS	NS	NS
Thigh muscle yield, %	42	23.79	22.05	23.13	23.23	23.14	23.8	23.73	0.323	0.815	NS	NS	NS	NS
Abdominal fat yield, %	42	2.20	2.11	2.06	2.08	2.05	2.02	2.00	0.027	0.526	NS	NS	0.047	NS

BW, body weight; ADG, average daily gain; ADFI, average daily feed intake; FCR, (feed intake : weight gain = g : g). a~d Within a row means with no common superscript differ significantly($p < 0.05$).[1]CON=basal diet; [2]PNM=basal diet containing 5,10,15% peanut meal; [3]FPNM=basal diet containing 5,10,15% fermented peanut meal; Orthogonal polynomials were used to determine linear and quadratic effect of peanut meal and fermented peanut meal. L=linear, Q=quadratic.

4.2 Meat quality

The impact of the treatments of PNM and FPNM on the breast meat quality of broilers at 42 d of age are shown in Figure 19. In terms of color (Figure 19A), the 10% FPNM group resulted in more red (higher a* value) meat when compared to the 10% PNM group. However, no significant differences were observed in lightness (L*) and yellowness (b*). The average pH (at 24 h from slaughtering) in the 10% FPNM group was significantly higher than that in the 10% PNM and CON groups ($p < 0.05$). Correspondingly, the ΔpH value in the 10% FPNM group was significantly lower than that in the other two groups (Figure 19B, $p < 0.05$). No significant differences were observed in shear force, cooking loss and drip loss among the groups (Figure 19C). In terms of the chemical composition (Figure 19D), the 10% FPNM birds showed a tendency of increased crude protein content in the breast muscle compared to the other two groups ($p = 0.071$), and there were no significant differences in moisture, fat and ash content. The oxidative stability of breast muscle are presented in Figure 19E. The DPPH scavenging activity was significantly increased in the 10% FPNM group compared to the CON group ($p < 0.05$), while there was no significant difference in ABTS scavenging efficiency. Compared to the broilers in the 10% PNM group, both the DPPH and ABTS scavenging activities were significantly increased in the 10% FPNM group ($p < 0.05$), but no significant difference was found between the 10% PNM and CON groups. Moreover, no significant differences were observed in the content of IMP in the breast muscle among the treatment groups (Figure 19F).

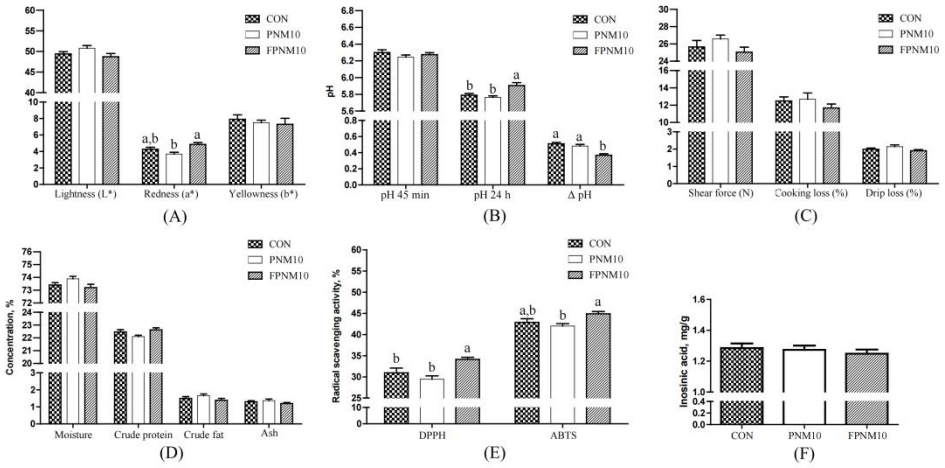

Figure 19. Effect of dietary PNM and FPNM supplementation on the breast meat quality of broilers (*n*=6). A, Meat color; B, The average pH at 45 min and 24 hour; C, The physical characteristics of breast meat; D, The

chemical composition of breast meat; E, The oxidative stability of breast meat; F, The content of IMP in the breast muscle.

4.3 Amino acid profile of the meat

The amino acid profile of breast muscle are shown in Table 24. Compared to the CON group, 10% PNM supplementation significantly decreased the levels of Lys, Met, Thr in the breast muscle ($p < 0.05$). Conversely, the level of Arg was significantly increased with 10% PNM supplementation ($p < 0.05$). In terms of the FPNM, the broilers showed significant increases in the levels of Glu, umami amino acids (UAA), Arg when compared with the CON group ($p < 0.05$). Furthermore, in comparison to the 10% PNM group, the 10% FPNM group exhibited significant increases in the levels of UAA, Thr, essential amino acids (EAA) ($p < 0.05$).

Table 24. Amino acid profile of breast muscle from broilers fed with PNM and FPNM supplemented diet (g/100 g meat, *n*=6)

Amino acids	Experiment treatment			SEM	*p*-value
	CON	PNM10[1]	FPNM10[2]		
Asp	2.30	2.18	2.27	0.030	0.237
Glu	3.31^{b}	3.45^{ab}	3.52^{a}	0.036	0.035
Umami amino acids[3]	5.61^{b}	5.63^{b}	5.80^{a}	0.035	0.045
Ser	0.88	0.86	0.85	0.014	0.681
Gly	1.00	0.96	0.99	0.018	0.582
Ala	1.13	1.10	1.13	0.027	0.894
Sweet amino acids[4]	3.01	2.91	2.96	0.032	0.487
Flavor amino acids[5]	8.61	8.54	8.76	0.047	0.157
Lys	2.24^{a}	2.08^{b}	2.16^{ab}	0.022	0.007
Met	0.90^{a}	0.80^{b}	0.86^{ab}	0.016	0.035
Thr	0.80^{a}	0.69^{b}	0.75^{a}	0.015	0.004
Leu	1.86	1.97	1.97	0.026	0.195
Ile	0.88	0.86	0.91	0.014	0.377
Arg	1.35^{b}	1.57^{a}	1.51^{a}	0.028	＜0.001
Val	0.82	0.75	0.77	0.017	0.221
Phe	1.05	1.03	1.03	0.016	0.791
His	0.86	0.92	0.91	0.015	0.222
Trp	0.26	0.25	0.26	0.006	0.516
Essential amino acids[6]	11.02^{ab}	10.90^{b}	11.21^{a}	0.052	0.033

Pro	1.05	0.98	0.96	0.021	0.219
Tyr	0.78	0.76	0.75	0.012	0.763
Cys	0.40	0.37	0.37	0.010	0.477
Total amino acids	21.84	21.55	21.97	0.078	0.063

[1]PNM10 = basal diet containing 10% peanut meal; [2]FPNM10 = basal diet containing 10% fermented peanut meal; [3]Umami amino acids = Asp + Glu; [4]Sweet amino acids = Ser + Gly + Ala; [5]Flavor amino acids = Umami amino acids + Sweet amino acids; [6]Essential amino acids = Lys + Met + Thr + Leu + Ile + Arg + Val + Phe + His + Trp; Mean values with different superscript letters within the same row are significantly different ($p < 0.05$), the results are represented as mean values with SEM.

4.4 Serum antioxidant performance

The effects of dietary supplementation of 10% PNM and 10% FPNM on serum antioxidant performance are summarized in Table 25. The T-AOC and SOD activities in the 10% FPNM group were significantly higher than those in the 10% PNM group ($p < 0.05$), but no significant differences were observed compared to the CON group. Correspondingly, the MDA content in the serum of the 10% FPNM group was significantly lower than that in the 10% PNM group ($p < 0.05$). In addition, there were no significant differences were observed in the CAT and GSH-Px activities among the groups.

Table 25. Effect of feeding PNM and FPNM on serum antioxidant performance of broilers

Items	Experiment treatment			SEM	*P*-value
	CON1	PNM10 1	FPNM10 2		
T-AOC, U/mL	9.9[a]	9.5[b]	10.2[a]	0.091	0.002
SOD, U/mL	165.7[ab]	158.8[b]	173.7[a]	2.109	0.006
CAT, U/mL	2.9	2.9	3.0	0.031	0.169
GSH-Px, U/mL	297.8	293.7	308.7	2.747	0.061
MDA, nmol/mL	6.1[b]	6.4[a]	6.2[b]	0.031	0.001

[1]PNM10 = basal diet containing 10% peanut meal; [2]FPNM10 = basal diet containing 10% fermented peanut meal. T-AOC, total anti-oxidant capacity; SOD, superoxide dismutase; CAT, catalase, GSH-Px, glutathione peroxidase; MDA, malonaldehyde; Mean values with different superscript letters within the same row are significantly different ($p < 0.05$), the results are represented as mean values with SEM.

5. Discussion

Fermentation has been shown to enhance the nutritional value of various feed ingredients, such as soybean meal and cottonseed meal, leading to improved growth performance in poultry, potentially attributed to increased digestibility of nutrients (Jazi et al., 2017b; Drażbo et al., 2019; Prematilaka et al., 2020a).

However, there is limited information available regarding the impact of FPNM on poultry growth performance. In the current study, 5%, 10%, and 15% PNM and FPNM were compared with the corn-soybean meal basal diet. The PNM groups showed a decreasing trend in BW and ADG, while FCR had an increasing trend. Especially, the growth performance of the group supplemented with 15% PNM was significantly lower than that of the CON group. Similar conclusions have been drawn in previous studies that when the proportion of PNM in the diet increases from 5% to 15%, even if the Lys and Met in the diet are sufficient, the growth performance and feed conversion rate of broilers will decrease (el Boushy and Raterink, 1989). Regarding various treatments with FPNM, there were no significant differences observed in BW of broilers at 21 and 42 d of age compared to the CON group, and the FCR was even better than that of the CON birds. Among them, the supplementation of 10% FPNM had the best effect. We believe that the improvement in growth performance is primarily associated with the following factors. Firstly, through fermentation, the levels of these anti-nutritional factors such as phytic acid can be significantly reduced, thereby improving nutrient utilization and promoting better growth in animals (Li et al., 2023c). Secondly, during the fermentation process, beneficial microorganisms produce enzymes that break down complex molecules, such as proteins and carbohydrates, into simpler forms. This enzymatic activity increases the digestibility and availability of amino acids, vitamins, and minerals present in the FPNM, which are essential for optimal growth and development in animals (Yafetto et al., 2022). Moreover, fermentation also contributes to the production of bioactive compounds and beneficial metabolites. These compounds, such as organic acids, enzymes, and probiotics, can positively influence the gut microflora and improve the gut health of animals (Zhu et al., 2020). A healthy gut environment enhances nutrient absorption, strengthens the immune system, and promotes overall growth performance. Slaughter performance includes indicators such as dressing yield, eviscerated yield, breast muscle yield, thigh muscle yield, and abdominal fat yield. These indicators serve as comprehensive measures to evaluate animal growth performance and economic efficiency, providing a direct reflection of the overall body composition and the proportion of edible portions in animals. In this experiment, dressing yields of all treatments exceeded 85%, while eviscerated yields surpassed 60%, meeting the standards for favorable meat production performance. However, no significant differences were observed among the groups. Previous studies have provided evidence that antioxidants play a role in reducing abdominal fat deposition in broiler chickens (Ashour et al., 2020). Interestingly, with the increase in FPNM supplementation levels, there was a linear decrease in abdominal fat yield, which may be attributed to the antioxidant effects exerted by FPNM.

The indicators for assessing meat quality include physical and chemical parameters such as color, tenderness, flavor, as well as chemical composition indicators (Faizur Rahman et al., 2020). In addition, the oxidative stability can affect the storage period and quality decline of meat, while IMP can influence the

flavor characteristics of meat (Yan et al., 2018; Xing et al., 2019). Meat color is an important parameter that provides valuable information about the freshness, appearance, and overall desirability of meat. The a* value measures the redness (+a*) or greenness (-a*) of the meat. Positive a* values indicate a more intense red color (Yang et al., 2022). In this study, we observed that the breast muscles from the 10% FPNM broilers exhibited higher a* values compared to the 10% PNM broilers. Studies have shown that higher a* values are often associated with increased oxidative stability and improved antioxidant activity in meat, and antioxidants play a crucial role in mitigating oxidative processes, thereby contributing to improved meat quality and prolonged shelf life (Chikwanha et al., 2019). Generally, higher pH levels contribute to the formation of a more stable myoglobin form, resulting in a more intense red color (Pogorzelska et al., 2013). This finding supports our research conclusion, as we observed higher pH values at 24 hours in the breast muscles of the 10% FPNM broilers. The chemical composition of meat is an important parameter that determines the quality and health benefits of poultry meat. In the study of Premathilaka et al., (2020b), the addition of 2% to 4% fermented soybean meal in the diet did not show any significant impact on the moisture, protein, fat, ash, calcium, and phosphorus content of the breast muscle. Similar conclusions were drawn in our study, where no significant effects on the chemical composition of the breast muscle were observed in both the 10% PNM and 10% FPNM birds. However, there was a tendency of increased crude protein content in the 10% FPNM group. We believe this improvement is associated with the increased efficiency of amino acid absorption after the fermentation of PNM (Li et al., 2023c). The DPPH and ABTS radicals are commonly used to evaluate the free radical scavenging ability of antioxidants (Zheng et al., 2020). Our study demonstrates that the 10% FPNM broilers' breast muscle exhibited a higher scavenging activity against DPPH and ABTS free radicals, indicating superior oxidative stability and consistent with the observed improvement in meat quality discussed earlier. Furthermore, IMP is an intermediate metabolite in adenosine triphosphate (ATP) metabolism and serves as an important indicator of the savory taste in chicken meat (Zhang et al., 2008). In this study, no significant effects were observed on the levels of IMP in the breast muscle with the addition of 10% PNM or FPNM.

The availability and proportions of different amino acids in the feed can influence the biosynthesis and incorporation of these amino acids into the breast muscle tissue (Macelline et al., 2021). In our study, we observed lower levels of Lys, Met and Thr in the breast muscle of broilers supplemented with 10% PNM. We attribute this to the relatively lower content of these amino acids in PNM (Li et al., 2023a). However, the breast muscle of broilers fed with 10% FPNM exhibited an improvement in amino acid levels, including a significant increase in essential amino acids compared to the 10% PNM group. This suggests an enhanced utilization of amino acids from FPNM, with some being deposited in the breast muscle tissue. Consistent with our previous findings, it has been demonstrated that the fermentation of PNM leads to a significant increase in the content of free amino acids (Li et al., 2023c). Free amino acids are known to exhibit higher efficiency of intestinal absorption compared to protein-bound

amino acids, as they do not require pre-digestion (Macelline et al., 2021). Additionally, the levels of Glu and umami amino acids were also increased, which contributes to the improvement of flavor characteristics in the breast muscle.

Oxidative stress is known to have a significant impact on the quality of meat. When broilers are exposed to oxidative stress, it can lead to the generation of reactive oxygen species (ROS) in the muscle tissue, these ROS can cause oxidative damage to lipids, proteins, and DNA (Zeitz et al., 2020). The oxidation of lipids leads to the development of off-flavors, rancidity, and discoloration in the meat. Additionally, oxidative stress can disrupt the structure and functionality of proteins, affecting their water-holding capacity, texture, and tenderness (Faustman et al., 2010). In the current study, we observed improved antioxidant capacity in the breast muscles of broilers fed with 10% FPNM, as evidenced by reduced MDA levels and increased activities of T-AOC and SOD. MDA, as the end product of lipid peroxidation, serves as a key indicator of oxidative stress (Geret et al., 2003). T-AOC represents the overall antioxidant capacity of enzymatic and non-enzymatic defense systems in the body (Birben et al., 2012). SOD, an important antioxidant enzyme, plays a vital role in scavenging free radicals and alleviating oxidative damage (Zhou et al., 2020b). The improvement in antioxidant capacity associated with FPNM may be attributed to two potential mechanisms. Firstly, the fermentation process of PNM leads to the formation of various fermentation by-products, such as organic acids and antioxidant peptides (Li et al., 2023c). Secondly, the antioxidative effects exerted by the bacteria involved in fermentation. In this study, it has been demonstrated that *Pediococcus acidilactici* LC-9-1, the strain used for fermentation, exerts antioxidative effects in broiler chickens by increasing the levels of total antioxidant capacity (T-AOC) and superoxide dismutase (SOD) in the serum (Li et al., 2023b).

6. Conlusion

The findings of the present study indicate that when PNM was added at a 5% proportion, there were no significant differences in growth performance compared to a conventional corn-soybean meal diet. However, when PNM was added at a 10% proportion, a decreasing trend in final BW was observed. Furthermore, when PNM was supplemented at a 15%, there was a significant reduction in growth performance. On the other hand, the dietary supplementation of FPNM (5%, 10% and 15%) demonstrated positive effects on broiler growth performance, antioxidant status, and meat quality. These effects were particularly notable when FPNM was supplemented at a 10% proportion of the diet.

Chapter VIII

General discussion, conclusions and perspective

1. Exploring the suitability of solid-state fermentation for PNM: a rational choice

1.1 Nutritional characteristics of PNM

PNM is a by-product obtained from peanuts after the extraction of oil through high-temperature pressing. It is commonly utilized as a feed ingredient due to its widespread availability and relatively low cost. However, there are several factors that limit PNM from being regarded as a high-quality feed ingredient.

First and foremost, PNM is characterized by an imbalance in amino acid composition. All our assessments are based on the amino acid requirements of broilers. Met, Lys, and Thr are the limiting amino acids in broiler nutrition and must be prioritized in diet formulation (Zhang et al., 2020). According to the optimal ratios of amino acids for chickens proposed by NRC (1994) and Texas A&M University He et al., (2021), the dietary requirements of Met, Lys and Thr for broiler chickens aged 1-21 days are 0.44%, 1.10%, and 0.74%, respectively. The measured content of these three amino acids in PNM was found to be 0.50%, 1.70%, and 1.28% (Table 26), whereas the corresponding content in soybean meal was 0.66%, 2.98%, and 2.02% (Cowieson et al., 2020). From this perspective, the amino acid composition of PNM is inferior to that of soybean meal. Interestingly, after fermentation, the content of these three amino acids in PNM changed to 0.59%, 2.05%, and 1.33%, respectively. Therefore, the use of FPNM is more favorable than PNM for formulating diets that meet the amino acid requirements of broilers. Additionally, Gly and Pro must be sufficient in poultry diets as chickens cannot synthesize them adequately in relation to their nutritional and physiological needs. The content of these two amino acids in PNM was 2.70% and 2.13%, respectively, and increased to 3.02% and 2.46% after fermentation, also showing some improvement. In addition, when compared to high-quality protein feed ingredients such as fish meal (Arg/Lys=0.76) and soybean meal (Arg/Lys=1.15), the ratio of Arg to Lys in PNM is typically between 3 and 4 (Li et al., 2023a). The excessive presence of Arg can result in amino acid antagonism within the body, hindering the absorption of Lys and negatively impacting animal growth performance (Zampiga et al., 2018). In addition, the bioavailability of amino acids needs to be considered. According to reports, soybean meal has been reported to have a standardized ileal digestibility rate of 90% for Met and 89% for Lys in broiler chickens (Chinese Feed Database News Web Center., 2020). In our study, the utilization efficiency of Met and Lys from PNM was found to be only 88% and 83%, respectively, which is significantly lower than that of soybean meal. This implies that even when the diet is formulated with normal amino acid levels, there may be a deficiency of essential amino acids deposited in the broilers' bodies. It is well understood that an excess supply of various amino acids in the diet does not necessarily mean improved animal nutrition, but a deficiency of amino acids can certainly impair growth performance and health in animals (Li et al., 2021). Therefore, from this perspective, the utilization efficiency of amino acids in PNM is also suboptimal.

Table 26. The amino acid requirements and optimal ratios for 1-21 d of age broilers

Items	Optimal ratios of amino acids (NRC, 1994 & He et al., 2021)[1]	Soybean Meal (Cowieson et al., 2020)	PNM (Chapter VI)	FPNM (Chapter VI)
Essential amino acids, %				
Met	0.44	0.66	0.50	0.59
Lys	1.10	2.98	1.70	2.05
Thr	0.74	2.02	1.28	1.33
Arg	1.15	3.68	5.12	3.29
Ile	0.74	2.11	1.64	1.73
Leu	1.20	3.52	3.02	3.34
Val	0.85	2.32	2.00	2.30
His	0.38	1.22	1.12	1.29
Phe	0.66	2.38	2.45	2.97
Non-essential amino acids, %				
Gly	1.94	2.17	2.70	3.02
Ser	0.76	2.72	2.15	1.85
Pro	2.02	2.37	2.13	2.46
Ala	1.12	2.11	1.91	2.63
Asp	0.73	5.91	5.43	5.69
Glu	1.96	8.44	8.97	9.48
Cys	0.35	0.65	0.55	0.62
Tyr	0.50	1.47	1.48	1.46

[1]The amino acid requirements for 1-21d of age broilers were calculated based on the recommendations provided by the NRC (1994) and the optimal ratios of amino acids for chickens by Texas A&M University (He et al., 2021).

Secondly, the presence of the anti-nutritional factor phytic acid (with approximately 1.5% content) can bind to proteins and reduce the utilization of PNM. It can also inhibit the activity of endogenous enzymes by binding to alkaline amino acid residues of pancreatic protease, thereby affecting nutrient absorption and impeding poultry growth (Nyman and Bjorck, 1989; Ren et al., 2017).

Additionally, PNM is susceptible to contamination by pathogenic microorganisms, including bacteria and fungi, which can cause diseases in animals.

1.2 Advantages of solid-state fermentation for PNM

To enhance the utilization efficiency of PNM in poultry diets, it is essential to

address the aforementioned challenges. Currently, research focused on improving the quality of PNM has made significant progress. Various methods have been explored, including physical, chemical, and biological approaches (Coomes et al., 1966; Prudente and King, 2002; Yang et al., 2016a; Zhao et al., 2023). While traditional physical and chemical methods are relatively simple to implement, they come with certain limitations. These methods may lead to nutrient losses in PNM, impact sensory quality, and introduce the risk of feed and environmental contamination through the use of additives. Consequently, the application of these methods is constrained. On the other hand, the application of biological processing methods holds greater promise.

A. **Fermentation can degrade peanut protein into peptides, thereby improving digestibility and absorption**. Research has shown that the absorption efficiency of peptides by animal intestinal epithelial cells is greater than that of free amino acids (Silk et al., 1979). Microbial fermentation of PNM can degrade high molecular weight antigenic proteins, resulting in FPNM with abundant plant peptides. This pre-digestion of PNM can enhance its digestibility and absorption in animals (Beuchat, 1976).

B. **Fermentation effectively removes antinutritional factors from PNM**. Noureddini and Dang (2010) used *Aspergillus usamii* to ferment soybean meal and found complete degradation of phytic acid. Animal experiments have demonstrated that fermentation significantly improves phosphorus utilization and reduces phosphorus excretion . Additionally, microbial degradation techniques for aflatoxin B_1 have been widely applied (Shu et al., 2018). In the current study, microbial fermentation of PNM can effectively reduce or eliminate phytic acid, crude fiber, and aflatoxin B_1, making it a promising protein source.

C. **Fermentation of PNM yields metabolites containing various beneficial factors**. During the degradation of high molecular weight proteins, fermentation generates numerous functional peptides and digestive enzymes. This not only improves the nutritional value of feed ingredients but also enhances feed digestibility and utilization (Gungor and Erener, 2020). Furthermore, microbial fermentation can accumulate beneficial metabolites and unknown growth factors (UGFs), regulating the microbial balance in the animal gastrointestinal tract and inhibiting the proliferation of pathogenic bacteria. It exhibits beneficial effects such as enhancing immune response, reducing diarrhea, and promoting growth, indicating the potential of microbial fermentation as a substitute for antibiotic additives (Wu et al., 2015).

D. **The metabolic activities of microorganisms during fermentation can produce various flavor compounds, including organic acids, enzymes, and extracellular polysaccharides**. These substances can enhance the palatability of feed, improve animal acceptance, and increase feed intake (Qiao et al., 2018).

2. Principles for selection of fermentation bacteria

According to the nutritional characteristics of PNM, we have established

principles for the selection of fermentation bacteria, including high acid production capacity, inhibition of pathogenic microorganisms, extracellular secretion of complex enzymes, and degradation of phytic acid. Choosing probiotic bacteria from the gastrointestinal tract of healthy animals offers the following advantages:

A. **Natural adaptability**: By selecting bacteria from the gastrointestinal tract of healthy animals, these strains already exist and reproduce in the intestinal environment of the animals, forming a symbiotic relationship with the host. Therefore, these strains are more likely to colonize and exert probiotic effects in the animal's digestive tract (Wang et al., 2021b).

B. **High survival ability**: Bacteria selected from the gastrointestinal tract of healthy animals generally possess higher survival abilities. They have adapted to the conditions of the animal's digestive tract, such as acidic environment, presence of bile salts, and competition from other microorganisms (Sathyabama et al., 2012). Therefore, these strains can better survive and colonize in the animal's intestines.

C. **Functional diversity**: Selecting Bacteria from the gastrointestinal tract of healthy animals allows for obtaining strains with diverse functionalities. Different probiotic strains may possess various probiotic characteristics, such as antimicrobial abilities, modulation of intestinal immune function, and enhancement of nutrient absorption (Petrof, 2009). Therefore, through diverse screening, it is possible to select probiotic strains that are suitable for specific animal requirements.

2.1 *Bacillus velezensis* LB-Y-1

Bacillus spp. has unique advantages as a fermentation feed bacterium.

A. **Heat and acid tolerance**: *Bacillus.* spp spores have strong tolerance and can survive and multiply in high-temperature and low-pH environments (Barbosa et al., 2005). This enables them to adapt to different environmental conditions during the fermentation of feed, maintaining the vitality of the microbial population.

B. **Increased feed nutritional value**: *Bacillus.* spp fermentation can degrade anti-nutritional factors in feed, such as phytic acid, cellulose, and non-starch polysaccharides, releasing potential nutrients (Hu and Kim, 2022). This improves feed digestibility and nutrient utilization, helping to enhance animal growth performance and health.

C. ***Bacillus* spp. possess high enzyme production capabilities, which is another important advantage in feed fermentation**. Deep fermentation capability: *Bacillus* spp. can produce various enzymes during fermentation, including proteases, cellulases, amylases, lipases, etc. These enzymes effectively degrade complex organic substances in feed into simpler forms, increasing feed digestibility and nutrient utilization (Latorre et al., 2016). Multifunctional enzyme system: Enzymes produced by *Bacillus* spp. often form complex enzyme systems with multiple enzyme activities. This multifunctional enzyme system can simultaneously degrade various complex feed components such as proteins,

cellulose, and starch (Cutting, 2011). This allows *Bacillus* spp. to perform multiple enzymatic functions in a single fermentation process, enhancing feed nutritional value.

In this study, we screened a strain of *Bacillus velezensis* LB-Y-1 from the gastrointestinal tract of healthy animals. This strain exhibits excellent potential for secreting extracellular complex enzymes (proteases, cellulases, and phytases) and also demonstrates the ability to degrade starch and fats. It is suitable for the fermentation of PNM.

2.2 *Pediococcus acidilactici* LC-9-1

Lactic acid bacteria are a widely used group of bacteria in solid-state fermentation.

A. **Improved feed digestion**: Lactic acid bacteria, through fermentation, produce lactic acid and other beneficial organic acids, which can lower the pH of the feed. Lowering the pH of the feed helps improve the digestion process and enhances the animals' utilization of nutrients in the feed (Dibner and Buttin, 2002).

B. **Antibacterial activity**: During fermentation, lactic acid bacteria produce beneficial antibacterial substances such as lactic acid, lactoperoxidase, and volatile fatty acids. These substances inhibit the growth of harmful bacteria, reduce the quantity of bacteria, molds, and other pathogenic microorganisms in the feed, thereby reducing the risk of microbial infections and improving feed preservation (Wu et al., 2018).

C. **Improved gut health**: Lactic acid bacteria can establish a beneficial microbial community in the animal's digestive tract, increasing the population of beneficial bacteria. These beneficial bacteria can competitively inhibit the growth of harmful bacteria and maintain a balanced gut microbiota (van Krimpen et al., 2021).

D. **Bio-safety**: The use of lactic acid bacteria is generally considered safe (GRAS) and do not exhibit significant toxicity or pathogenicity to animals and humans (Lee et al., 2022).

In the current study, we selected *Pediococcus acidilactici* LC-9-1, which demonstrated excellent acid production efficiency, antimicrobial properties, and antioxidant characteristics. Furthermore, both in vitro and in vivo safety assessments were conducted for this strain.

3. Evaluation of nutritional changes and application effects of FPNM in broilers

PNM underwent fermentation using the aforementioned bacteria and conventional solid-state fermentation techniques. Significant improvements were observed in the levels of crude protein, TCA-soluble protein, and L-lactic acid. Additionally, notable reductions were observed in the concentrations of crude fiber, phytic acid, and aflatoxin B_1. These changes were achieved through the use

of conventional solid-state fermentation techniques, which have been widely employed in the fermentation of plant protein sources (Wang et al., 2020). In the future, further optimization of the fermentation process is expected to result in even greater nutritional improvements in PNM. The observed changes indicate that the selected bacteria successfully established stable populations in PNM and exerted their effects through the metabolic activities. It should be noted that the increase in crude protein content is primarily associated with a decrease in the concentration of non-structural carbohydrates. This reflects a reduction in the actual dry matter content, as we did not add additional nitrogen sources during the fermentation process, resulting in a nominal increase in protein content (Wu et al., 2020).

Furthermore, fermentation significantly enhanced the apparent ileal digestibility (AID) and standardized ileal digestibility (SID) of essential amino acids, such as Met, Lys, Leu, and Phe, in broilers. This improvement may be attributed to the increased levels of free amino acids and peptides in FPNM after fermentation, indicating an improved protein hydrolysis process. As a result, the higher proportion of soluble low-molecular-weight proteins facilitated easier protein absorption by the chickens, leading to improved amino acid utilization (Freitas et al., 2011). In this study, no significant impact on the apparent metabolizable energy (AME) between PNM and FPNM was observed, which is consistent with the findings of (Li et al., 2022b). Fermentation is a metabolic process in which some carbohydrates in PNM are initially utilized by microorganisms, resulting in energy loss during this process (Song et al., 2014). However, fermentation also converts some indigestible fibers into new carbohydrates. Furthermore, changes in protein composition can affect energy metabolism. Smaller protein molecules often have simpler structures and shorter amino acid sequences, requiring less energy during degradation and absorption in the gastrointestinal tract (Gilbert et al., 2008a). Additionally, fermentation microorganisms can utilize low-quality proteins in PNM to synthesize microbial cell proteins. It is known that microbial cell proteins can be effectively utilized by broilers, and their biological value is similar to that of soybean meal (Freitas et al., 2011). In conclusion, the energy metabolism of FPNM is influenced by various factors, including alterations in carbohydrates, fiber, protein composition, anti-nutritional factors, total energy, and other metabolic by-products. Therefore, different fermentation processes can yield varying results in terms of increased, decreased, or maintained metabolic energy of the fermentation substrate.

Fermentation has been demonstrated to enhance the nutritional value of various feed ingredients, such as soybean meal and cottonseed meal, resulting in improved growth performance in poultry (Jazi et al., 2017b; Drażbo et al., 2019; Premathilaka et al., 2020a). However, limited research has focused on the impact of FPNM on poultry growth performance. Therefore, the objective of our last study was to investigate the effects of FPNM on the growth performance, meat quality, and oxidative stability of broiler chickens compared to diets containing corn-soybean meal and PNM. The results indicated that broilers fed diets containing 10% FPNM showed significantly higher body weights and average

daily gain compared to the PNM groups. Additionally, the 10% FPNM group exhibited the lowest feed conversion ratio. This finding suggests that FPNM can positively impact the economic aspects of broiler production by reducing feed costs and improving production efficiency. In terms of meat quality, broilers in the 10% FPNM group displayed improved meat color (a* value), $pH_{24\ hour}$ values, and oxidative stability of the breast muscle compared to the 10% PNM group. The addition of 10% PNM resulted in decreased levels of essential amino acids (Lys, Met, and Thr) in the breast muscle, while the inclusion of 10% FPNM mitigated these changes. Furthermore, the 10% FPNM group showed enhanced antioxidant capacity, with higher levels of T-AOC and SOD, and lower levels of MDA, indicating reduced oxidative stress compared to the 10% PNM group.

Importantly, we cannot overlook the role of the two bacterial strains, LB-Y-1 and LC-9-1, used for fermentation. By measuring the bacterial content in FPNM, we determined that their respective levels were 1.1×10^6 CFU/g and 2.3×10^4 CFU/g. The concentration of LC-9-1 was lower than that of LB-Y-1, which we speculate might be due to losses during the drying (48℃) process, as *P. acidilactici* is unable to form spores like *Bacillus.* spp, limiting its ability to withstand adverse conditions (Yang et al., 2019b). Additionally, in section 4.6 of chapter IV, we assessed the impact of LB-Y-1 on broiler growth performance with a concentration of 3.5×10^6 CFU/g in the experimental diet. The study revealed that LB-Y-1 enhanced digestive enzyme activity and increased levels of alkaline phosphatase and albumin in the serum, resulting in improved growth performance and tibia mineralization in broilers. The added concentration was comparable to the level of LB-Y-1 found in FPNM, providing reason to believe that it played a positive role in FPNM. In section 4.6 of chapter V, we evaluated the influence of LC-9-1 on broiler growth performance, using a concentration of 5.5×10^6 CFU/g in the experimental diet. The research demonstrated that LC-9-1 improved the antioxidant capability and intestinal innate immunity of broilers, leading to enhanced growth performance and reduced abdominal fat percentage. Although the concentration of LC-9-1 in FPNM is much lower than the level of 5.5×10^6 CFU/g, we believe that it still has a positive impact on the health of broiler chickens. This may be due to the fact that probiotics primarily exert their effects through metabolites within the host's body (Husted et al., 2017; Huo et al., 2022). During the process of solid-state fermentation, a significant accumulation of metabolites, including lactic acid, occurs in FPNM. When consumed by broiler chickens, these metabolites can act more quickly and exert beneficial effects, similar to the effects of direct supplementation with LC-9-1.

In Figure 20, we have presented a comprehensive summary of the effects and underlying mechanisms associated with the incorporation of FPNM in broiler diets. Our analysis suggests that the observed enhancements in meat quality and antioxidant oxidative stability can be attributed to two primary factors. Firstly, probiotics and their metabolites can enhance carcass traits and meat quality in poultry. Studies by Endo and Nakano (1999) demonstrated that supplementing broiler diets with a combination of probiotics (*Lactobacillus* spp., *Bacillus* spp., *Clostridium* spp. etc.) improved carcass characteristics and meat quality. A

research from Liao et al., (2015) also showed that adding *Clostridium butyricum* to broiler diets improved breast muscle yield and meat color. Secondly, fermentation products such as amino acids play a significant role in enhancing meat quality and oxidative stability. Fermentation of PNM resulted in a 37.7% increase in Ala content (from 1.91% to 2.63%), and improved the apparent ileal digestibility in broiler chickens by 3.9% (from 83.51% to 86.80%). Ala, the simplest natural β-amino acid, has been shown to significantly reduce MDA levels and increase glutathione peroxidase (GSH-Px) activity in broiler breast muscles when added at a concentration of 0.12% in the diet (Mannion et al., 1992). Additionally, Ala can increase the muscle peptide content, attenuate lipid oxidation, and reduce stress induced during feeding, thus enhancing the antioxidant capacity of the body (Smith et al., 2012). Moreover, fermentation of PNM resulted in an 11.85% increase in Gly content (from 2.70% to 3.02%) and improved the apparent ileal digestibility in broilers by 7.34% (from 67.45% to 72.40%). Gly possesses antioxidant and cytoprotective properties, and can alleviate heat stress-induced dysfunction of antioxidant status and intestinal barrier in broilers (Deng et al., 2023). Furthermore, Glutathione (GSH), composed of Glu, Cys and Gly, is an important component of the body's antioxidant defense system (Rodriguez et al., 2019). In our study, the apparent ileal digestibility of these amino acids was significantly increased, which enhanced the antioxidant capacity of the body.

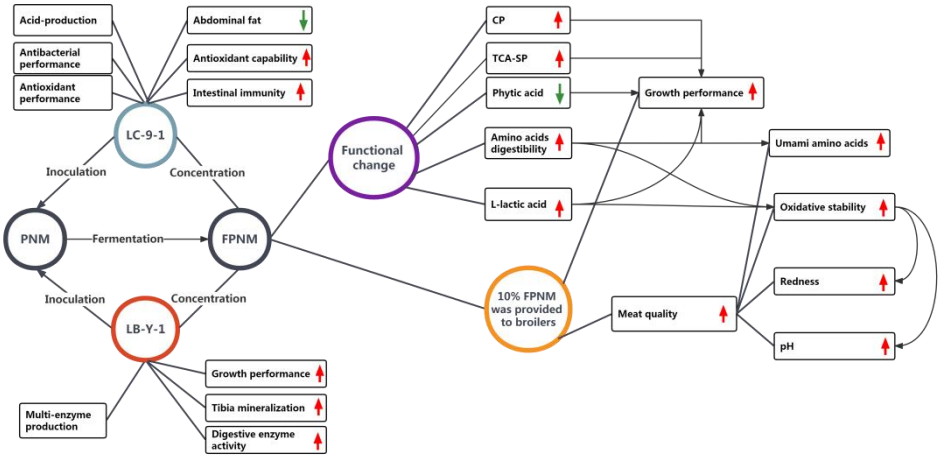

Figure 20. Summary of the application effects and potential mechanisms of FPNM supplementation in broiler diets

4. Cost-effectiveness

In addition, we conducted a rough cost analysis of FPNM production. This is beneficial in determining whether the cost reduction associated with this product in broiler chicken production is significant. It is important to note that the

fermentation process not only consumes energy but also incurs losses in dry matter (Hu et al., 2015). By conducting weight measurements before and after fermentation under consistent moisture conditions, we determined that there was a dry matter loss rate of 6.3% during the fermentation process of PNM (data not shown). The observed dry matter loss rate is consistent with the findings of previous studies on the fermentation of rapeseed meal conducted by Wu et al. (2020). This means that 1.07 tons of PNM is required to produce 1 ton of FPNM.

Considering the average selling price of PNM in the Chinese market in 2022, which was $401.57 per ton (Source: http://www.huasheng7.com). The rough cost of producing 1 ton of FPNM is as follows: $429.68 (1.07 tons of PNM cost) + $11.43 (starter culture) + $13.57 (steam sterilization) + $4.28 (first-stage fermentation under quasi-aerobic conditions) + $5.7 (re-mixing) + $3.57 (second-stage fermentation under quasi-anaerobic conditions) + $30.71 (drying) + $12.86 (labor cost), totaling $511.80 per ton. This cost is advantageous compared to the average selling price of soybean meal in 2022, which was $642.85 per ton (Source: https://finance.sina.com.cn/money/future/wemedia/2023-02-08/doc-imyeynxs6437643.shtml). In the experiment conducted in Chapter VII, when 10% of the corn-soybean meal diet was replaced with FPNM, no significant differences in feed intake were observed, and there was a trend towards improvement in final BW. Overall, the cost analysis suggests that the production of FPNM through the described process is advantageous compared to soybean meal from a cost perspective. These findings contribute to the understanding of the economic feasibility and potential benefits of incorporating FPNM into broiler diets.

5. General conclusion

A. Based on the nutritional characteristics of PNM, we screened two bacteria for PNM fermentation through a specific screening process, namely *Bacillus velezensis* LB-Y-1 and *Pediococcus acidilactici* LC-9-1. The former demonstrated good acid production efficiency, antimicrobial activity, and antioxidant properties, which can reduce abdominal fat deposition in broilers and enhance their innate immune response in the intestines. The latter exhibited excellent potential for producing complex enzymes, thereby improving broiler growth performance and tibial mineralization.

B. PNM was subjected to fermentation employing the above-mentioned bacteria and conventional solid-state fermentation techniques. Significant enhancements were observed in the levels of crude protein, TCA-soluble protein, and L-lactic acid levels. Moreover, notable reductions were observed in the concentrations of crude fiber, phytic acid, and aflatoxin B_1. Furthermore, the apparent ileal digestibility (AID) and standardized ileal digestibility (SID) of essential amino acids such as Met, Lys, Leu, and Phe in broilers were significantly improved after fermentation. However, no significant impact on the apparent metabolizable energy (AME) between PNM and FPNM was observed.

C. Feeding trial was conducted by adding different gradients of PNM and

FPNM (5%, 10%, and 15%) to broiler diets. The results indicated that compared to corn-soybean meal diets, the impact of PNM on growth performance decreased linearly with increasing addition levels. On the other hand, FPNM positively influenced broiler growth performance, meat quality, and oxidative stability, with the optimal performance achieved at an addition level of 10%.

6. Perspectives

A. Current studies have demonstrated the positive impact of solid-state fermentation on the enhancement of nutritional value in PNM. However, there is still room for improvement in the fermentation process. For instance, optimizing fermentation temperature, duration, moisture content, and bacterial inoculum through gradient experiments may lead to FPNM with higher nutritional value.

B. FPNM has shown positive effects on meat quality improvement, but the underlying mechanisms remain unclear. It is necessary to identify the new metabolites or fermentation products generated during the process. Investigating these aspects is an interesting and extensive task. Once these questions are addressed, we can make more targeted adjustments to the purpose and process of fermentation, allowing for a more precise control of fermentation to meet specific goals.

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Appendices

Scientific publications

Scientific publications

Adatpted From the Thesis:

1. **Li C**, Li S, Zhu Y, *et al*. Improving the nutritional value of plant protein sources as poultry feed through solid-state fermentation with a special focus on peanut meal—advances and perspectives [J]. ***Fermentation.*** 2023, 9(4):364. (**Chapter II**)

2. **Li C**, Li S, Dang G, *et al*. Screening and characterization of *Bacillus velezensis* LB-Y-1 toward selection as a potential probiotic for poultry with multi-enzyme production property [J]. ***Frontiers in Microbiology.*** 2023, 17(4): 1143265. (**Chapter IV**)

3. **Li C**, Wang S, Chen S, *et al*. Screening and characterization of *Pediococcus acidilactici* LC-9-1 toward selection as a potential probiotic for poultry with antibacterial and antioxidative properties [J]. ***Antioxidants***, 2023, 12(2): 215. (**Chapter V**)

4. Li S†, **Li C**†, Chen S, *et al*. Effects of solid-state fermentation on the standardized ileal digestibility of amino acids and apparent metabolizable energy in peanut meal fed to broiler chickens[J]. ***Fermentation***, 2023, 9(4):346. (**Chapter VI, Co-first authors**)

Related topic:

1. **Li C**, Cai H, Li S, *et al*. Comparing the potential of *Bacillus amyloliquefaciens* CGMCC18230 with antimicrobial growth promoters for growth performance, bone development, expression of phosphorus transporters, and excreta microbiome in broiler chickens [J]. ***Poultry Science***, 2022, 101(11): 102126.

2. **Li C**, Li S, Liu J, *et al*. *Escherichia coli* O88 induces intestinal damage and inflammatory response through the oxidative phosphorylation and ribosome pathway in Pekin ducks [J]. ***Frontiers in Cellular and Infection Microbiology***, 2022, 8: 1187.

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