

Probiotics formulated from indigenous bacteria from goat digestive tract and fed with fermented mixed feed *Eichhornia crassipes* and *Zea mays* cobs

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Abstract. Isnawati, Muhaimin FI, Rahayu DA, Fitrihidajati H, Ratnasari E. 2023. Probiotics formulated from indigenous bacteria from goat digestive tract and fed with fermented mixed feed *Eichhornia crassipes* and *Zea mays* cobs. *Biodiversitas* 24: 2906-2911. The provision of cellulolytic bacteria as probiotics in goat feed is crucial to their growth and health. Therefore, this study aimed to monitor the effects of providing cellulolytic bacteria isolated from the digestive tract of goats as probiotics in feed rations. Probiotic cellulolytic bacteria were isolated from the digestive tract of goat food fed with a fermented feed containing a combination of hyacinth and corn cob. In this study, the probiotic types are also different from other studies, namely *Bacillus pumilus*, *Bacillus brevis*, and *Pseudomonas diminuta*. Therefore, its effects on the vegetative and generative growth from various age groups and sexes of test goats are investigated. The goats tested were divided into two groups: fed with and without probiotics, with subgroups based on their sexes (male and female) and age groups (baby, juvenile, and adult). The tested goats were fed for 35 days, and their weight gain and sperm quality were observed; in general, weight gain was observed in goats fed the probiotics. Furthermore, the quality of spermatozoa in adult male goats fed with probiotics is higher than without probiotics, which includes its viability (52.2% to 36.9%), motility (48.1% and ++++ to 33.2% and ++), volume and concentration (1.05 mL/ejaculate with 3.7×10^9 cells/mL to 0.55 mL/ejaculated with 2.6×10^9 cells/mL). Based on the results, adding cellulolytic bacterial probiotics to the feed promotes the vegetative growth of goats in different age groups, in male and female goats. Also, it improves reproductive performance in adult male test goats.

Keywords: Cellulolytic bacteria, corn cob, feed, synbiotics, water hyacinth

Abbreviations: LAB: Lactic acid bacteria, NPBM: Non-probiotic baby male, NPDF: Non-probiotic baby female, NPTM: Non-probiotic teen male, NPFT: Non-probiotic teen female (NPFT), NPAM: Non-probiotic adult male, NPAF: Non-probiotic adult female, WPBM: With probiotic baby male, WPMF: With probiotic baby female, WPJM: With probiotic juvenile male, WPJF: With probiotic juvenile female, WPAM: With a probiotic adult male, WPAF: With probiotic adult female

INTRODUCTION

Feed is one of the most important elements in achieving optimal livestock productivity, affecting the growth stage and quality of life, including reproductive ability. Therefore, selecting the right type is an effort to increase livestock growth rate and feed efficiency (Beigh et al. 2016). Furthermore, increasing goat productivity requires an adequate supply of quality feed with good nutritional value that must be cheap, accessible, and available all year round. Legume is commonly used as a fodder due to its advantages such as low reliance on fertilizer nitrogen (N) inputs, high voluntary intake, and high protein content which increased food intake as well as good conversion efficiencies. Legume also improves production and reduces environmental impacts, enhancing energy and protein use efficiency in ruminants due to its bioactive content, such as condensed tannins (Phelan et al. 2014; Lagrange et al. 2021). Unfortunately, the cultivation of legumes is threatened by possible climate change (Kulkarni et al. 2018).

Rapid investigations and development to improve livestock management and operational efficiency have been continuously carried out in the agricultural industry, including animal feed. There are several types of feed: fermented feed, plant-based feed (calliandra mixture, mahogany, cacao, rambutan, jackfruit, cassava leaves) and wheat bran (polar). Subsequently, feeding 50% fermented feed and 50% factory animal feed resulted in the highest weight gain in Etawa crossbreed goats compared with 100% fermented feed and 100% factory feed (Pakpahan and Restiani 2019). Other studies used fermented feed made from water hyacinths called *fermege* in goats. It has been reported to have high nutritional content and provide many benefits to the goat, such as increasing goat weight, enhancing the quality of carcasses with high protein content, reducing the manure odor, increasing the female cubs, and enhancing the sperm quality of male goat's, as well as sheep's (*Ovis aries* Linnaeus 1758) (Fitrihidajati and Isnawati 2017; Ratnasari et al. 2018; Isnawati et al. 2021). That might result from the fermentation process of microorganisms, which degrade complex compounds on

the feed raw material into simple compounds that are easier to digest. In addition, microorganisms that enter the digestive tract of goats along with the feed can function as probiotics, which positively impacts their health.

The use of probiotics, prebiotics, and synbiotics to improve human health quality has been widely practiced (Markowiak and Slizewska 2018). Microorganisms used as probiotics can fight pathogenic microorganisms found in the gastrointestinal tract, suppress the growth of harmful microorganisms, accelerate the process of food digestion, improve the structure of the gastrointestinal tract, and increase the absorption rate of nutrients (Mingmongkolchai and Panbangred 2017; Zokaeifar et al. 2014; Jorgensen et al. 2016). However, it can also be used on targeted livestock, particularly mammals similar to human which its digestive system that utilize microorganism to breakdown complex carbohydrate to simple sugar especially cellulose. Therefore, it is essential to study the production of probiotics, prebiotics, and synbiotics to increase the goats' breeding efficiency.

Unlike humans, where most probiotics belong to the group of lactic acid bacteria (LAB), probiotics for ruminant animals are mainly cellulolytic bacteria such as *Enterobacter*; It is a group of cellulolytic bacteria can degrade microcrystalline cellulose in culture media (Li et al. 2014). Hendraningsih and Wahyudi (2022) investigated the use of cellulolytic bacteria isolated from rumen fluid as probiotics. Furthermore, previous studies examined the use of several probiotics (*Lactobacillus*, *Enterococcus*, *Streptococcus*, *Propionibacterium*, and *Prevotella bryantii*) and yeast (*Saccharomyces* and *Aspergillus*) (Kulkarni et al. 2022). In addition, using cellulolytic bacteria isolated from buffalo rumen has been administered to increase egg production and cholesterol production performance in egg yolk (Candrawati et al. 2017).

This study was designed to evaluate the effects of probiotic feed derived from cellulolytic bacteria from the goat digestive system on the weight of male and female goat samples. In this study, the probiotics were *Bacillus pumilus*, *Bacillus brevis*, and *Pseudomonas diminuta* isolated from the goat's digestive tract from its feces which then added to the fermented feed. Previous observation has shown the goats which were previously fed with the fermented feed with a mixture of water hyacinth and corn cobs, has an increasing meat protein content and spermatozoa quality (Fitrihidajati and Isnawati 2017; Ratnasari et al. 2018). In addition, this study evaluated the spermatozoa quality of male goats fed with probiotic feed.

MATERIALS AND METHODS

Animals

Domestic goats (*Capra hircus* Linnaeus 1758) aged four, seven, and 12 months old were used as experimental animals. The tested goats received approval from the health research ethical clearance commission of Universitas Airlangga, Faculty of Dental Medicine (Decision no: 683/HRECC.FODM/VIII/2022) to experiment as test animals. This study was conducted at the goat farm in

Tegalrejo, Jombang, East Java, Indonesia, for 40 days in the August-September of 2021.

Bacteria isolation from goat feces

In this study, 1 g of feces was suspended in 10 mL of sterile water, vortexed for ≥ 60 min, and rested. The suspension was diluted 10-fold from 10^{-1} to 10^{-6} to prepare a bacterial stock. Then, the bacterial stock was cultured on nutrient broth solid media and incubated at 30°C for 24 h. After 24 h, a single colony was streaked on the same media agar plate and incubated at 30°C for 24 h. Finally, isolated strains were cultured, maintained, and stored in a nutrient broth media with 5% glycerol media at 4°C to collect bacteria cultures from various environments.

Bacteria characterization and identification

Probiotic bacteria are selected based on cellulolytic activity, low pH, and bile salts tolerance. Therefore, bacteria were selected as probiotics with high cellulolytic activity (which shows a clear zone on CMC media), acid-fast, and bile salt-resistant. The isolated bacteria were molecularly analyzed based on the 16S rRNA gene sequence, and Basic Local Alignment Search Tool (BLAST) was used to determine the bacterial species.

Feed additive probiotic studies

A total of 24 male and female goats were divided into 12 subgroups. The first six groups were fed without probiotics: Non-probiotic baby male (NPBM), Non-probiotic baby female (NPBF), Non-probiotic teen male (NPTM), Non-probiotic teen female (NPTF), and Non-probiotic adult male (NPAM), Non-probiotic adult female (NPAF). Next, the following six subgroups were fed with additional probiotics: with probiotic baby male (WPBM), with probiotic baby female (WPMF), with probiotic juvenile male (WPJM), with probiotic juvenile female (WPJF), with probiotic adult male (WPAM), and with probiotic adult female (WPAF). The feed provided was a mixture of conventional feed, chopped/cogon grass (*Imperata cylindrica* (L.) P.Beauv.), and kale (*Ipomoea aquatica* Forssk.) with a ratio of 1:1. The probiotics added consisted of *B. pumilus*, *B. brevis*, and *P. diminuta*, with 1:1:1 ratio in the form of 1% of probiotic powder, and the administration was carried out in 35 days. *B. pumilus*, *B. brevis*, and *P. diminuta* were isolated from goat feces previously fed with fermented feed containing water hyacinth and corn cobs mixed for 90 days. In addition, the experimental parameters include the body weight in all test goat groups and the spermatozoa quality in adult males, measured weekly for five weeks.

Spermatozoa quality measurement

Therefore, to determine the quality of the spermatozoa from male goats fed with and without probiotic feed or drinking water, semen was collected using an artificial vagina. Subsequently, observation parameters include individual and mass of spermatozoa, viability, concentration, semen volume, color/consistency, and pH. Furthermore, 200 spermatozoa were stained with eosin to obtain viability and then observed with a light microscope

with 400x magnification. The dead spermatozoa would be red, while the live ones would be colorless. The motility percentage of individual spermatozoa was observed using a light microscope with 400x magnification (Ax et al. 2000). The percentage of the motility of mass spermatozoa criteria and calculation of the semen concentrations are based on the procedure by Susilawati et al. (2021).

Statistical analysis

Data were analyzed using the general linear model procedure of IBM SPSS Statistics 26 (SPSS Inc., Chicago, Illinois, USA). In addition, body weight data were analyzed using a one-way ANOVA test with significance at the 0.05 probability level, and the spermatozoa quality was analyzed by descriptive analysis.

RESULTS AND DISCUSSION

The weight gain of the goats fed with probiotics

Livestock growth can be assessed by observing the increasing height, length, circumference, and body weight in healthy animals given adequate food, drink, and shelter. The potential growth of livestock is determined by genetics, which translates into hormonal relationships in the body, resulting in differences in growth rate and adult weight achieved (Alemneh and Getabalew 2019). This study compares the economics of rearing goats with two treatments: feeding with and without probiotics. During 35 days of observation, weight was measured weekly, and it was observed that the weight gain and difference of goats aged four months in males and females with probiotic feed was higher than the weight gain of goats without probiotic feed (Figure 1). Moreover, the weight gain and difference of 7 and 12 months old goats, both males and females, with probiotic feed were also higher than those without probiotics (Figures 2 and Figure 3).

Probiotics are more commonly used than antibiotics due to their benefits in maintaining healthy and fast-growing livestock. The use of probiotics has been shown to enhance gut health and growth performance, maintain a balance in gut microbiota, stimulate immunity against pathogenic organisms, and improve digestion as well as overall production efficiency in ruminants, poultry, and swine production (Lambo et al. 2021). Antibiotic substitutes have improved health and growth in weaned goats (Zhang et al. 2020).

In this study, the probiotics used included *B. pumilus*, *B. brevis*, and *P. diminuta*. *B. pumilus* has been shown to regulate microbial communities by increasing the abundance of beneficial bacteria, decreasing pathogenic bacteria in the rumen and caecum, and promoting healthy conditions (Zhang et al. 2020). *B. brevis* has potential as a probiotic because it produces antibiotic peptides gramicidin S (Berditsch et al. 2007), brevibacillin, and bogorol that have antibacterial, antifungal and anti-invertebrate agents

(Yang and Yousef 2018). These compounds can inhibit and even kill bacteria, fungi, and invertebrates that are pathogenic and detrimental to livestock. Therefore, giving *B. brevis* as probiotics to the ruminant can increase the vegetative growth of livestock. Meanwhile, *P. diminuta*, also called *Brevundimonas diminuta*, has been shown to secrete extracellular enzymes with proteolytic activity. Two proteases were detected migrating at 67 kDa and 50 kDa. Both hydrolyzed gelatin preferentially, but casein was also degraded, and slight hydrolysis was observed with hemoglobin (Adriano et al. 2000). The optimal temperature and pH for proteolytic activity were between 40°C and 50°C, with a pH ranging from 7.0 to 11.0, respectively. Because of their proteolytic activity, these bacteria can be used as ruminant probiotics, which aid in the digestion of proteins in the digestive tract of goats.

The quality of spermatozoa

The viability of spermatozoa was observed by eosin staining; dead spermatozoa were bright, while live spermatozoa were colorless (Khan et al. 2017; Tanga et al. 2021). Furthermore, it was observed that the spermatozoa viability of goats fed with a probiotic ration was better than those without a probiotic feed ration. These met the nutrition required for optimal spermatogenesis and improved epididymis conditions to maintain spermatozoa's viability (Khan et al. 2017). Probiotic activity in feed can increase the protein content, and it was important in supporting the optimal spermatogenesis process and good epididymis conditions. The changes in feed, mainly in nutritional content, affect the quality of the sperm produced by sheep and goats. The nutrient content significantly affects the seminiferous tubules' size and spermatogenesis quality (Martin et al. 2010). A high protein content feed resulted in a high level of luteinizing hormone (LH) and blood testosterone, which play critical roles in spermatogenesis and viability. It also caused the complementary organs in the spermatogenesis process to work optimally to produce seminal fluid with the right compound. This is crucial in maintaining normal metabolic processes in spermatozoa and supporting sperm viability (Ramaswamy and Weinbauer 2014).

The motility of spermatozoa is crucial because it determines success in reaching the egg in the fallopian tubes. This study covered the motility of individual and mass spermatozoa, and the motility of the individual spermatozoa of the goats fed with a probiotic ration was better than without a probiotic ration. The probiotic activity gives feed the best total digestible nutrient (TDN) that meets the precursor compounds needed for spermatozoa motility (Johnson et al. 2020). Subsequently, fructose is the primary source of carbon and energy necessary for the motility of spermatozoa, which can be produced from various other forms of carbohydrates. In addition, sorbitol, glyceryl-phosphorylcholine (GPC), and plasmalogens are energy sources to support its movement and survival.

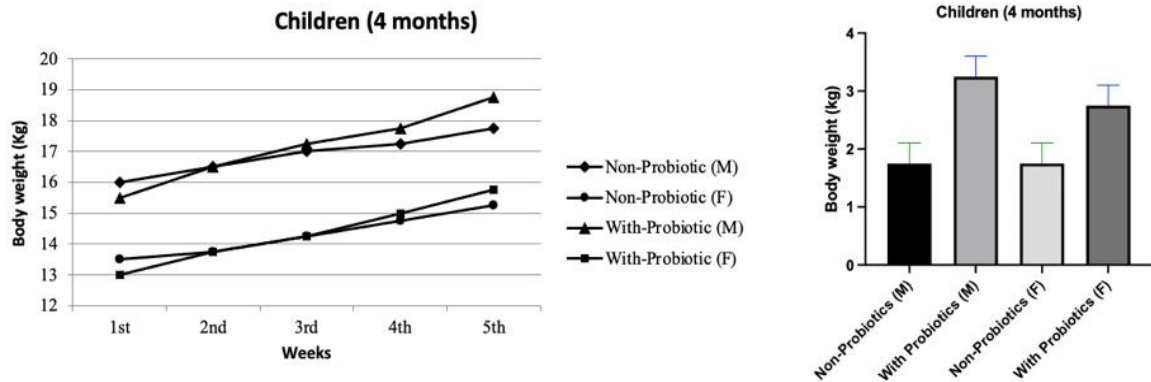


Figure 1. The weight gain and differences of four months old goats (n=8)

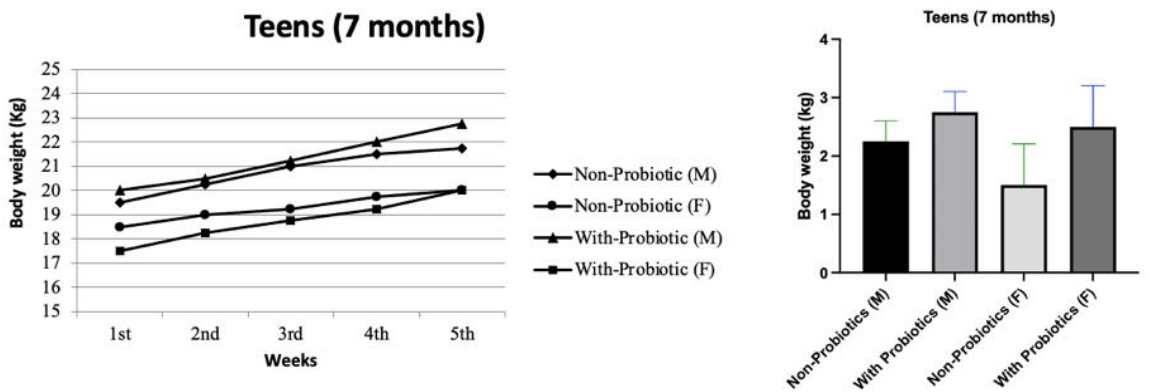


Figure 2. The weight gain and differences of seven months old goats. (n=8)

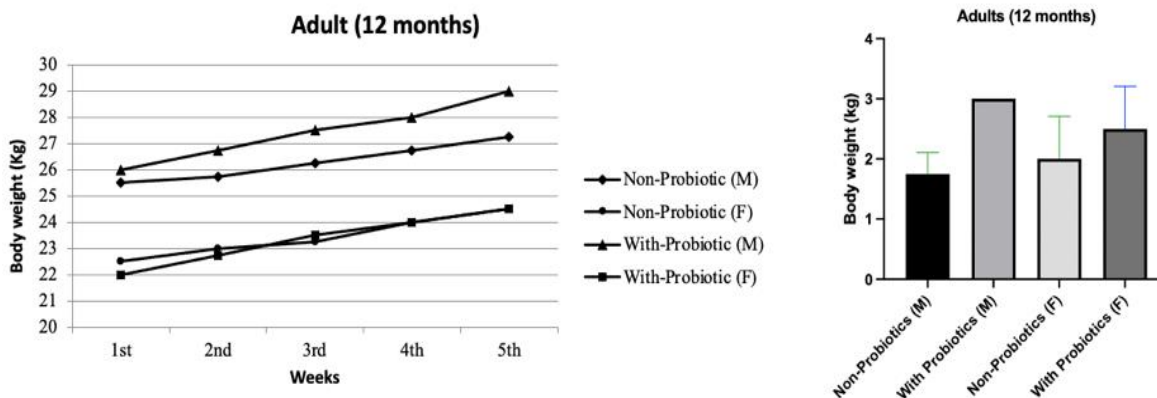


Figure 3. The weight gain and differences of 12 months old goats. (n=8)

The mass movement of spermatozoa is the accumulation of individual movements. Furthermore, it formed large, enormous, dark, thick, and bouncy waves like a plume of black clouds before the rain moved quickly (Susilawati 2021; Pamungkas et al. 2014). The results showed that the mass movement of spermatozoa of goats fed with probiotic feed had a higher velocity than those

without probiotic feed (Table 1). Moreover, the highest concentration of spermatozoa was obtained from goats fed with probiotic feed. Therefore, the test's spermatogenesis process mainly determined the spermatozoa concentration. Several factors significantly influence the process of spermatogenesis, including genetic factors, hormonal conditions, and food consumption.

Table 1 Comparison of the sperm quality of goats fed with and without probiotic feed

Parameters	Without probiotic feed	With probiotic feed
Spermatozoa viability (%)	36.9	52.2
Individual spermatozoa motility (%)	33.2	48.1
Mass spermatozoa motility	+ / + +	+ + +
Spermatozoa concentration (cell/ml x 10 ⁹)	2.6	3.7
Semen volume from one-time ejaculation (mL)	0.55	1.05
Semen color/consistency	Milky white	Cream
Semen pH	6.84	6.86

The formation of key regulators in the testicular tissue, such as cAMP-responsive element binding protein (CREB) and cAMP-responsive element modulator (CREM), will trigger spermatogenesis. CREB molecules are required to control spermatogenesis (Walker et al. 1995). CREB group transcription factors are needed to regulate gene expression to respond to several signaling pathways (Fimia et al. 1999). The CREM transcription factor is expressed directly inside the testicles and plays a role in the maturation of spermatozoa (D'Aurora et al. 2017). CREM proteins play an essential role in the haploid germ cell differentiation of males (Behr and Weinbauer 1999). The study by Blendy et al. (1996) showed that a lack of CREM would affect the expression of protamine, leading to infertility due to spermatid abnormalities in mice. The formation of the components involved in spermatogenesis certainly comes from feed consumed by livestock, which is further processed in their bodies to form reproductive hormones. These different types of reproductive hormones significantly affect the production and quality of spermatozoa (Rahayu et al. 2017).

The amount of semen released during ejaculation can be influenced by several factors such as species, body weight, testicular size, age, feed, and frequency of ejaculation (Suyadi et al. 2020). Livestock such as goats and sheep generally produce low volumes of semen, while cattle generally with larger testes leading to higher semen volume. However, the volume of semen does not determine the ability of spermatozoa to fertilize the egg successfully. The feed ratio with probiotics resulted in the highest volume of semen, indicating that feed greatly affects the volume of semen. In this study, the range volume of semen obtained was in line with the opinion of Susilawati et al. (2021), who reported that the volume of sheep spermatozoa ranges from 0.5-2.0 mL.

The color of the semen observed ranged from milky white to cream. That aligns with the opinion of Ax et al. (2000) and Susilawati et al. (2011) that the color of goat and sheep spermatozoa ranges from milky white to light beige. The color of the semen is influenced by the number of spermatozoa it contains. The more the number of spermatozoa are, the darker the color of the semen (close to cream). Meanwhile, the degree of acidity (pH) dramatically affects the viability of spermatozoa. The degree of acidity (pH) of semen varies depending on the species of cattle. Chaudhari et al. (1990) stated that semen with a higher presence or concentration tends to have a slightly acidic pH. The sperm of sheep/goats have a pH ranging from 6.4-

7.2, or an average of 6.8 (Hahn et al. 2019). In this study, the two feed rations produced relatively the same pH, namely 6.8.

In conclusion, this study reported that goats fed with fermented water hyacinth and corn cobs had improved vegetative growth in all groups (children, teens, and adults). In addition, it also increased spermatozoa quality microscopic and macroscopically due to the presence of cellulolytic bacteria in their digestive tract acting as probiotics.

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