Dynamics of the Bacterial Community in Fermentation Process of Rice Straw as Animal Feed

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Abstract. Rice straw is a potential fiber forage as feed for ruminant. The aim of this research was to observe the potential of Stimulator Plus on improving the nutritional quality of fermented rice straw. The material that has been used in this study were rice straw, stimulator plus, starbio, urea, and molasses. Completely Random Design has been applied in this experiment, with four treatments fermentation period (0, 7, 14, and 21 days) with three replications, Proximate Test and Van Soest to determine the nutrient content of fermented products. The Next-Generation Sequencing (NGS) method for metagenomic analysis of a microorganism. The observed variables were physical quality and chemical quality. Fermentation period at seven days increased the crude protein content and decreased the Neutral Detergent Fiber and acid detergent fiber of fermented rice straw (P<0.01). The bacterial diversity in fermented rice straw was measured using some indices: Chaó1 index varied from 1717 to 2599; Shannon index varied from 20.66 to 23.72; Simpson index varied from 4.086 to 4.813; Abundance-based Coverage Estimator (ACE) index varied from 31.916 to 34.838; Se.ACE index varied from 1987.61 to 2905.7. The Bacillaceae and Streptococcaceae were always found throughout the fermentation. Bacterial community dynamics were found different in each treatment.

Keywords: dynamic, bacterial community, fermentation process, rice straw, animal feed

1. Introduction

Agricultural by-product, such as rice straw, is common in tropical area as ruminant feed. However, this kind of feed has been known for low nutrient content that leads to low animal production [1]. Rice straw has a low protein (2-6% crude protein) but has a lot of raw fiber (lignin, cellulose, and hemicellulose), with 40 - 48% of total digestive nutrition (TDN) [2]. The structure of rice straw passes through the lignification process to form lignocellulose and lignocellulose which are difficult to digest [3].

Stimulator Plus is one of the fermented additives containing a mixture of microorganisms. The stimulator plus is made of cow rumen containing cellulolytic bacteria. Stimulator Plus at dose 10% showed an increasing on nutrition quality of fermented rice straws [4]. However, there was limited information on optimum fermentation period with Stimulator Plus. The common fermentation...
additive that has been known for years were Starbio and urea with 21 days of fermentation. The addition of Stimulator Plus gives a good prospect to accelerate it.

Next-Generation Sequencing (NGS) is one of the culture-independent technique for the microbial community metagenomic analysis. Application of NGS have resulted in the significant progress in medical microbiology for metagenomic analysis of pathogenic microorganism, the causal agents of infectious diseases [5];[6]. A similar study was conducted using NGS techniques to analyze the microbial communities in the fermented food such as Korean soybean paste, doenjang [7];[8]. In this study metagenomic analysis in fermented rice straws using the NGS method to reveal the microbial diversity involved in the fermentation process was conducted. This research aimed to observe the potential of Stimulator Plus on improving the nutritive quality of fermented rice straw at different fermentation period.

2. Materials and Method

This research used completely randomized design with four treatments of fermentation periods; i.e. 0, 7, 14, and 21 days with three replications in each sample. The 5 kg of rice straws in the fresh matter (FM) were placed into plastic bags, then added 5 g of urea + 5 ml of molasses + 5 ml of Stimulator Plus + 5 g of Starbio, then added water to obtain 60% of moisture content. Then air was manually removed from the bags and tied tightly with rope. The observed variables were physical quality including odor, color, texture, and the existence of fungi as well as the chemical quality including crude protein, dry matter, neutral detergent fiber (PDF) and acid detergent fiber (ADF). Total genome DNA from samples was extracted using ctab/SDS method. DNA concentration and purity were monitored on 1% agarose gels. According to the concentration, DNA was diluted to 1ng/μl using sterile water. 16s rRNA genes of distinct regions were amplified used a specific primer with the barcode. All PCR reactions were carried out with phusion® high-fidelity PCR master mix (new England Biolabs). Mix same volume of 1 x loading buffer (contained syb green) with PCR products and operate electrophoresis on 2% agarose gel for detection. Samples with a bright main strip between 400-450bp were chosen for further experiments. PCR products were mixed in equidensity ratios. Then, mixture PCR products were purified with Qiagen gel extraction kit (Qiagen, Germany). Sequencing libraries were generated using truseq® DNA PCR-free sample preparation kit (Illumina, USA). The library quality was assessed on the qubit® 2.0 fluorometer (Thermo scientific) and Agilent bioanalyzer 2100 system. At last, the library was sequenced on an illuminahissequ2500 platform and 250 bp paired-end reads were generated [9].

The nutrient content of fermented rice straw was analyzed by analysis of variance (ANOVA) to observe the effect of treatment with the dependent variable. Duncan Multiple Range Test (DMRT) is applied to observe differences between treatments [10]. Bacterial dynamics were analyzed by the NGS method by combining paired readings and quality control: Data solving, Merging reading, Data filtering, Chimera removal. The tag is finally obtained. OTU cluster and Species annotation; OTU production, Species annotation, Construction of phylogenetic relationships, Normalization Data. Alpha diversity for samples through indexes; Chao1, Shannon, Simpson, ACE, good coverage. All of these indications are calculated with QIIME (Version 1.7.0) and displayed with R software (Version 2.15.3) [9].

3. Results and Discussion

3.1. Dynamics of bacteria on fermentation process

The diversity of bacterial communities in fermented rice straw samples in variations of 0, 7, 14 and 21 days was analyzed at each classification level (kingdom, phylum, class, order, family, genus, and species). The dominant phylum found in fermented rice straw samples was identified as twelve major
dominant bacterial families and represented 77.3% of the total bacterial population. *he Bacillaceae and Streptococcaceae was always found throughout the fermentation.*

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\textbf{Table 1. The family of bacterial population} \\
\begin{array}{ll}
\text{Family} & \text{Percentage (%)} \\
\text{Bacillaceae} & 26.4 \\
\text{Streptococcaceae} & 17.2 \\
\text{Staphylococcaceae} & 9.2 \\
\text{Planococcaceae} & 6.1 \\
\text{Enterobacteriaceae} & 5.0 \\
\text{Clostridiaceae} & 4.8 \\
\text{Streptomycetaceae} & 2.8 \\
\text{Micrococcaceae} & 2.6 \\
\text{Pseudomonadaceae} & 1.1 \\
\text{Xanthomonadaceae} & 0.8 \\
\text{Dermabacteraceae} & 0.7 \\
\text{Flavobacteriaceae} & 0.6 \\
\end{array}
\]

The rarefaction curves indicated how large fraction of the species diversity had been discovered. All the curve becomes flattered to the right, indicating that most OTUs in the sample was detected and analyzed. There were 1,783 OTUs found on day 0 fermentation, on the 7th day 1,425 OTUs, 1,879 OTUs were found on the 14th day and 1,498 OTUs on the 21st day. There are 138 OTUs found at each stage of fermentation.

![Figure 1. Rarefaction curve](image-url)
In this study, alpha diversity was used to analyze the variety of both the richness and abundance of bacterial species in 0, 7, 14 and 21 days fermented rice straw samples. Based on the analysis of alpha diversity in the sample calculated based on ≥97%, the homologous sequences were obtained in the form of Chao1 index varied from 1717 to 2599; Shannon index varied from 20.66 to 23.72; Simpson index varied from 4.086 to 4.813; Abundance-based Coverage Estimator (ACE) index varied from 31.916 to 34.838; Se.ACE index varied from 1987.61 to 2905.74 (Table 1).

Table 2. Alpha Diversity Index of rice straw fermented with Stimulator Plus

| Fermentation Period (day) | Chao1   | Shannon | Simpson | ACE     | Se.ACE     |
|---------------------------|---------|---------|---------|---------|------------|
| 0                         | 1717    | 20.661  | 4.086   | 32.199  | 1987.61    |
| 7                         | 2096    | 22.050  | 4.403   | 34.838  | 2391.80    |
| 14                        | 2599    | 23.725  | 4.813   | 31.915  | 2905.74    |
| 21                        | 2363    | 23.428  | 4.488   | 34.568  | 2690.53    |

The Chao1 and ACE indices describe the richness of bacterial species found in fermented rice straw samples in variations of 0, 7, 14 and 21 days. Shannon and Simpson's index shows the diversity of bacterial species in a community found in fermented rice straw at 0, 7, 14 and 21 days of fermentation. The index value showed that the diversity of bacterial species found in fermented rice straw in this study is categorized in the medium diversity level [11].

At the beginning of fermentation, the family of *Staphylococcaceae* and *Enterobacteriaceae* were dominant. Then after 21 days fermentation by the bacteria family *Streptococcaceae* and *Bacillaceae* were dominant (Figure 2).

![Figure 2. Distribution of bacterial families in rice straw fermented with Stimulator Plus](image)

Along with the process of fermentation at 7, 14 and 21 days fermentation is dominated by the bacteria family *Streptococcaceae* and *Bacillaceae*. Found five species from the family Bacillaceae,
namely: Bacillus cereus, Bacillus firmus, Bacillus flexus, Bacillus ginsengihum, Bacillus clausii, Bacillus firmus. And found species from the family Streptococcaceae, namely Lactococcus garvieae. Other than found 2 species from the family Staphylococcaceae, namely: Staphylococcus sciuri, Staphylococcus aureus.

Bacillus spp. classified into the class of heterotrophic bacteria, ie protists are unicellular, belonging to the class of reducers microorganisms or commonly referred to as decomposers. Most marine bacteria included in the group of bacteria are heterotrophic and saprophytic [12]. The genus Bacillus is a rod-shaped bacterium that can be found in soil and water, including in seawater. Some types produce extracellular enzymes that can hydrolyze proteins and complex polysaccharides. Bacillus spp forms endospores, is gram-positive, moves in the presence of the periodic flagellum, can be aerobic or facultative anaerobic and catalase positive [13].

3.2 Physical and chemical properties of fermented rice straws

Physical quality of fermented rice straw from 0 until 21 days was shown in. The pH value of fermented rice straws was 7.07 at 0 days, then decreased to 5.51 at 21 days (Table 3). It showed that there were no significant differences in pH value from 7 days until 21 days of fermentation period. [14] reported that there are some changes on fermentation process i.e water soluble carbohydrates will be fermented by homofermentative bacteria resulting lactic acid or by heterofermentative bacteria resulting lactic acid, acetic acid, ethanol, and CO₂. Fermentation process decreased pH value (lactic acid bacteria would decrease pH value quickly). Color, odor, texture and existence of fungi were in the same condition until 21 days of fermentation.

The different superscripts on the same line show significant differences (P<0.01)
The chemical quality of fermented rice straw from 0 until 21 days was shown in Table 4.
Table 4. Chemical quality of rice straws fermented with Stimulator Plus

| Variables (%) | Fermentation period (day) | 0     | 7     | 14    | 21    |
|---------------|---------------------------|-------|-------|-------|-------|
| Dry matter (DM) |                           | 23.92^a | 20.37^b | 19.18^b | 22.22^b |
| Crude protein (CP) |                         | 3.35^b  | 8.07^a  | 10.08^a | 8.32^a |
| NDF           |                           | 73.05^a | 47.07^b | 47.60^b | 42.96^b |
| ADF           |                           | 58.96^a | 40.90^b | 41.35^b | 41.53^b |

The values followed by different superscripts on the same line show significant differences (P<0.01)

Fermentation process decreased dry matter content (P<0.01). It can be explained that during the fermentation process, microorganism degrades the nutrient content and producing organic acids [15]. Prolonging the fermentation process from 7 days to 21 days did not affect the DM content of rice straw silage (P>0.05). This stagnant DM content was affected by the acidity of rice straw. In this study, pH value was not different at 7 to 21 days of fermentation. Therefore the DM content showed no clear changes. The low pH value in silage, prevent the undesirable microorganism compete with lactic acid bacteria for nutrient [16]. This result in agreement with [17] that reported rice straw fermented with fermented juice of lactic acid bacteria increased the crude protein content of rice straw silage. According to [15], during the ensiling process protein were breakdown to peptides, amino acids and NH₃. Fermentation period at 7 days showed degradation (P<0.01)of NDF and ADF content. There were no significant different at 7, 14 and 21 days fermentation on NDF and ADF content. It can be suggested that microorganism in Stimulator Plus has an ability for fiber degradation. The possibility of the presence of microorganism is selulolitic bacteria since the one of the ingredient of Stimulator Plus was rumen liquid.

Obtained bacterial dynamics from the Bacillaceae family which experienced an increase in abundance. This has to do with the ability of these bacteria such as proteolytic and cellulolytic. Overall, using of stimulator plus on fermentation period 7 days could increased physical and chemical quality of fermented rice straws including decreased of pH value, NDF and ADF content and increased of crude protein content and dry matter. Prolonging the fermentation period from 7 to 21 days did not show any differences or advanced increases. The bacteria found in each treatment showed differences and diversity.

4. Conclusion.

The Bacillaceae and Streptococcaceae were always found throughout the fermentation. Bacterial community dynamics were found different in each treatment.

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