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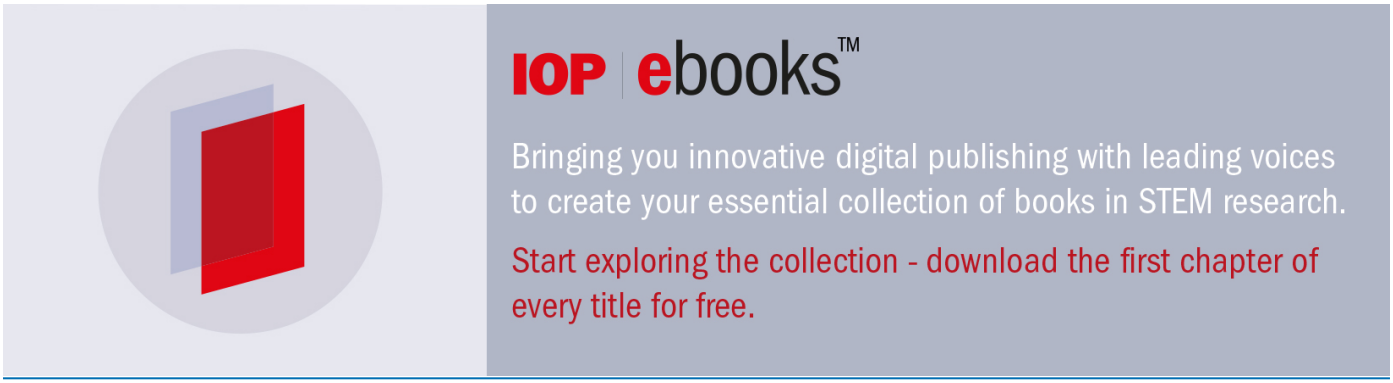

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Molecular Diversity of Lactic Acid Bacteria on Ileum and Coecum Broiler Chicken Fed by *Chrysonilia crassa* Fermentation

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Abstract. The Lactid Acid Bakteria (LAB) are microflora in the digestive tract which has positive roles in poultry's health. One of the factors diversity of LAB in the gatrointestinal tract are influenced by feeding factor. The purpose of this study was to analyze the LAB diversity in ileum and coecum after being fed on fermented *Chrysonilia crassa* molecularly. LAB species diversity was analysed to provide a baseline profile of the microbial community database on the ileum and coecum digestive tract of broiler chicken of control (commercial feed) and treatment (feed with *Chrysonilia crassa* fermentation) by the method of Terminal Restriction Fragment Lenght Polymorphism The calculated values werethe number of phylotypes, relative abundace, Shannon-Wiener diversity index (H'), evennes index (E'), and similarity. Group of LAB detected in the control group were *Lactobacillus delbrueckii* (180 bp), *Lactobacillus* sp. (187 bp), *Lactobacillus plantarum* (572 bp), *uncultured bacterium* (87 bp) and *unidentified* (50 bp, 582bp). The result of this study showed that by feeding on the fermented *Chrysonilia crassa* feed had resulted in the decreasing of LAB diversity, i.e. ileum (0.66), coecum (0.48) compared with commercial feed (control) that was ileum (0.84), coecum (1.05).

Keywords: broiler chicken, coecum, ileum, LAB, T-RFLP

1. Introduction

One of source of animal protein which is widely consumed by the public is broiler chicken meat because beside conataining high nutritious food, broiler chicken costs affordably. Based on the data of Central Statistics Office in 2016, the consumption of broiler in 2015 reached 0.103 kg per capitain a week [1]. Public interest in the consumption of broiler chicken were being targeted by the animal husbandry industry to increase their productivity. One of optimization attempts that was done is to maintain the condition of the digestive tract to keep the chicken healthy.

One of the caracteristics healthy chicken is its healthy digestive tract. It means that the condition of the chicken's digestive tract is stable and free from intestial inflammation which is caused by the presence of pathogenic bacterial population, feed antigens and toxics within the lumen of the small

intestine [12]. The part of digestive tract which indicates the healthy digestion is the ileum and coecum. Ileum is essential to digestion and absorption of various types of macromineral and essential nutrients contained in the feed [18]. Coecum or seka is a unique feature of the poultry digestive tract. This part is located on the border between the small and large intestines. Coecum is a place absorption proceeds and the fermentation procees which is carried out by microbes and then will produce some of vitamin B [13].

One of alternatives that has been carried out to improve the quality of feed ingredients in chicken is the technique of solid substrate fermentation. Fermentation feeding is assumed to be able to repair the existing microflora on chicken digestive tract especially lactid acid bacteria (LAB) [20]. *Chrysonilia crassa* fungus is used to ferment feed of rice bran. *Chrysonilia crassa* fungus is a filamentous fungus that has potential as a probiotic in vitro. Probiotic which is derived from fungi are expected to maintain the resilience and stability during feed manufacture and viability during transit thourgh the gastrointestinal tract of chicken [21].

The diversity of LAB in the chicken's digestive tract, particularly in the ileum and coecum will affect the health of the animal. The type of LAB after being given *Chrysonilia crassa* fermented feed has been analyzed to provide initial profile in the form or database on the diversity of LAB as the constituent of microbial commuity.

2. Materials and Method

2.1. Sampling

Samples of lumen content on ileum and coecum were obtained from Laboratory of Physiology and Biochemistry, Faculty of Agriculture and Animal Science of Diponegoro University. The samples used in this study were the lumen part of the ileum and coecum of broiler chicken by the addition of *Chrysonilia crassa* fermented as the treatments and commercial feeding as the control with the method of five repetition be used once per chicken.

2.2. Isolation of Genomic DNA Directly from the Sample

Isolation of DNA genomic directly from sample by metagenomical approach based on Jannah (2014). A total 0f 0.25 g luminal contents of the sample parts of the ileum and coecum were washed with 500 µL of PBS buffer (Phosphate Buffer Saline, pH 7.2) by centrifugation 13.000 rpm for 5 minutes (Zhu *et al.,* 2002). The DNA of the bacterial genome was extracted by Mini Kit from Mo Bio in accordance with company instructions. Each sample was given extraction of bacterial genomic DNA and also in isolates *of Lactobacillus salivarus* marker. The quality and purity of the DNA isolation results was being measured using a DNA analyzer spectrophotometer.

2.3. Amplification and Purification of 16S rRNA Gene

The process of 16S rRNA gene amplification using 7F FAM forward primer which was abeled with 6-carboxyfluorescein (5' - AGAGTTTGATCCTG GCTCAG - 3') and specific reverse primer for lactic acid bacteria, named primary SG-LAB-0677 (5'CACCGCTACACATGGAG-3') which were not labeled (Heilig *et al*., 2002; Dicksved *et al.,* 2007). PCR reaction mixture consisting of 25 µL GoTaq *GreenMaster Mix* (Promega, USA), 2 µL of each primer (10 pmol), and *destilated water* and 100 ng of template DNA in a final concentration until a final volume of 50 µL. PCR Condition was performed with an initial denaturation at 95°C for 5 minutes, followed by 30 cycles of denaturation at 95°C for 40 seconds, *annealing* temperature 52°C for 40 seconds and the temperature of the extension at 72°C for 1 minute, then final elongation at 72°C for 7 minutes[4].

The PCR products were confirmed by electrophoresis using 1% agarose gel in 1 x TAE buffer and visualized by ethidium bromide (EtBr) pigmentation using *Gel Documentation System* (atto Corporation, Japan). PCR products were then purified by GeneJet™ PCR *Purification Kit* (Fermentas, USA) in accordance with the instructions of the company. DNA which was obtained from refining was then added 40 µL nuclease- *free water* and stored at -20°C until the further use.

2.4. Restrictions / cutting PCR Products with Restrictions Enzymes

The restriction of PCR products with the restriction enzyme was carried out based on [8], the purified PCR product was cut with the restriction enzyme of *Hae* III and *Msp* I (Fermentas, USA). Each restriction consists of 15 units of the restriction enzyme (1.5 µL) of 10X restriction buffer (2 µL, R buffer for enzyme of *Hae* III and Tango buffer for enzyme *Msp* I) and 100 ng of DNA samples (10 mL) then was added with *Nuclease-free water* until the final volume up to 20 mL. The cutting PCR product DNA using the restriction enzyme of *Hae* III and *Msp* I was for detecting polymorphisms 16S rRNA gene sequences [8]. Subsequently the samples were incubated at 37^0C for 16 hours, and deactivated with a temperature of 80^0C for 20 minutes and immediately cooled in an *ice bath* based on the instructions of company. Restriction product was precipitated by ethanol precipitation and was added with 3 µL of Nuclease free water.

2.5. The Analysis of TRFLP

The resulting products from the restriction was sent to a service company Fragment Analysis 1 st base (http://www.base-asia.com/fragment_analysis/) Malaysia to be analized its capillary electrophoresis. The conditions of reaction used to determine the cut length of PCR products labeled *fluorescence* substance was the results of DNA samples with restriction enzyme cutting that had been purified in 1 µL added with a mixture of 10 ml of formamide and internal standard (Gene Scan - 500 ROX, Applied Biosystems) (100: 5, vol / vol). The pieces of T-RF labeled was analyzed using electrophoresis with *automatic sequence analyzer* (ABI PRISM 3100, Applied Biosystems) in the gene scan mode, and the length of the T-RF was determined by comparing the *Peak ScannerTM vl software. 0* (Applied Byosystems). The obtaining results then were matched by using database (http://mica.ibest.uidaho.edu/) [8].

T-RF with difference chemical base size less than 0.5 bp on the same sample were cut by using same enzyme then were classified as the same T-RF [5]. The T-RF size was then rounded to the nearest tens. Furthermore, the T-RF with the area less than 1% of the T-RF total on the same sample was considered as impurity, as a result it was not used in the analysis. To predict the *phylogenetic* affiliation T-RF size appearing in the community, it was carried out by finding its similarity with the size of T-RF bacteria existed in the database of *Ribosomal Database Project* (RDP) using program *Microbial Community Analysis* 3 (MiCA3) *Virtual Digest* (ISpaR) accessed on (http://mica.ibest.uidaho.edu/digest.php). The same T-RF size as the T-RF size in the database was predicted have the same *phylogenetic* relationship.

2.6. The Data Processing

The data processing using T-RFLP method was based on fluorescence signal. To distinguish between the fluorescence signal and noise then the specified threshold in which for further analysis used the value of T-RF ≥ 50 bp and the percentage area of peak (peak) ≥1% [10].

The results obtained were in the form of decimal fractions and then rounded to the nearest value, and presented in the percentage value. Those then were presented into table of binary data (indicating presence or absence of T-RF), in which peaks with a percentage of the total <1% were categorized as 0 (none) and the percentage of the total area of ≥ 1% were categorized as 1 [2]. The value of diversity or diversity of bacteria was the number of phylotype in the bacterial communities [4]. Phylotype richness (S) is totally different peak TRF / types of different restriction found in each sample. The value of biodiversity is determined by:

a. The number of phylotype found in the sample (S)
b. The Shannon-Wiener diversity index (H ') were calculated to illustrate the diversity of the community on different instar and the relative importance value in each *phylotype* in over all the community using the following formula: $H' = - \Sigma$ pi ln (pi), in which *pi* is the sample size owned by *Phylotype* (pi = ni / n) [16]

c. The evenness index was determined by the level of community spread of the T-RF community. Evenness *(evenness)* was calculated using the community of *Pielous* index *(J)* as follows: E '= H' / ln (S)
   S was the total number of T-RF [16]
d. The similarity index / similarity pair Sorensen's (Cs) was calculated for each pair of the community with the following formula: Cs = 2J / (a + b), in which J was the number of T-RF / *phylotype of* every compared community, a and b are the total number of each T-RF / *phylotype* each community, the index value in range of 0-1, the value of 0 means no *phylotype* overlapping between communities, the value of 1 is contained exactly the same *phylotype* found in both communities.
e. The determination of specific *phylotype* number found in a community.

Each T-RF was considered as one *phylotype* (can also be considered as a species). Those *phylotype* identifications were used by isolates bacteria *of L. salivarius* CSP004 as isolates marker which then was used as a standard for analysis of lactic acid bacteria (LAB), which then was cut with the same restriction enzyme in the sample and observed its size T-RF.To identify the T-RF results, program T-RFLP analysis ISPAR (*Insilico* PCR and Restriction) of MiCA3 *(Microbial Community Analysis)* using RDP *database* (R10, U270 consisting of 1.519.356 references of bacteria based on 16S rRNA gene were used. The obtaining results then will be matched using database of MiCA3 (http://mica.ibest.uidaho.edu/).

3. RESULT AND DISCUSSION

3.1. DNA Extraction and Amplification of the Lumen Ileum and Coecum Content Samples Broiler Chicken Sorts

DNA extraction was performed at the beginning stage in this study of DNA extraction on the contents of the lumen ileum and coecum broiler chicken. DNA derived from the contents of the lumen ileum and coecum broiler chickens in this study was used as a DNA template in amplifying the 16S rRNA gene region. From the visualization of 16S rRNA gene amplification results, it was obtained 650 bp DNA band on each sample. It showed that the amplification results were based on the positive control *of Lactobacillus salivarius*[8].

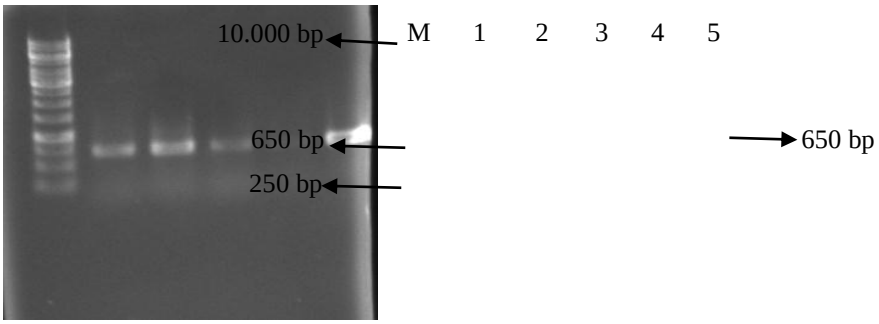

Figure 1. Electrophoresis DNA Amplification results from the sample luminal contents of the ileum and cecum part of broilers in 1% agarose gel with marker 1 kb (1 = control ileum, cecum 2 = control, 3 = treatment ileum, cecum 4 = treatment, 5 = *L. salivarius).*

3.2. Purification and Restriction of DNA Fragment

The concentration of the purified DNA obtained from the microbial community control and treatment were in the ranges from 36-68 ng/ml and A260/A 280 obtained ranged between 1.74-1.89. Based on the results obtained after purification that the concentration of DNA was known to be higher than the result of DNA extraction, this was because at the purification stage, the DNA was purified and separated from the contaminant components (such as RNA, protein and other components remaining) higher. The concentration and purity produced had a good quality to continue the process of digestion using restriction enzymes.

3.3. The community of Bacteria and T-RF Profile

The result of T-RFLP analysis in the form of T-RF data of BAL community was shown in the form of electroferogram. In reading the data on the display was by using Peak Scanner elektroferogram. The LAB results in the ileum and coecum of chicken both by controling and treating using restriction enzymes *Msp*1.

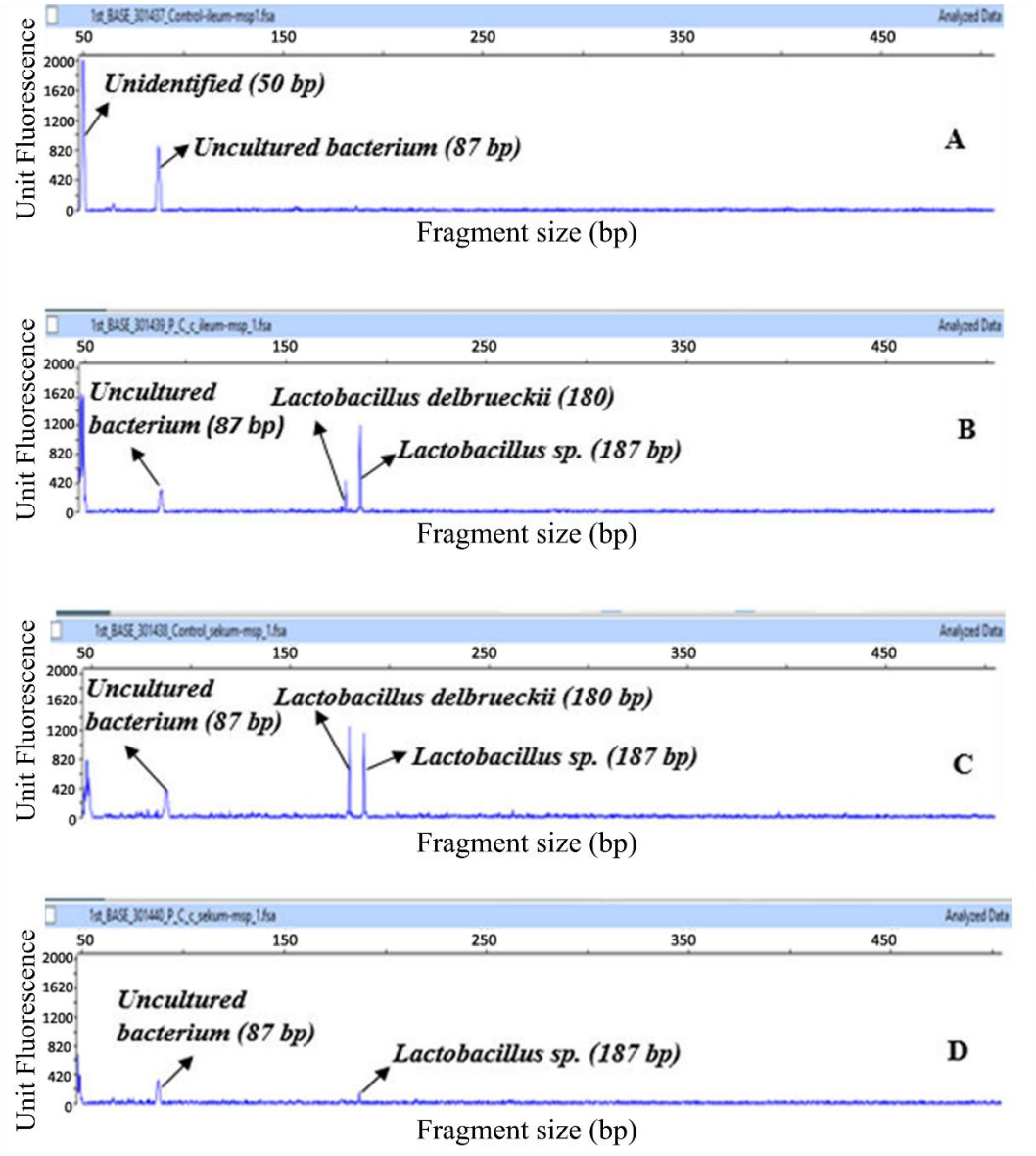

Figure 2. The LAB community T-RF electroferogram pattern on ileum and coecum of broiler chicken using restriction enzyme *Msp*1: (A) Control in ileum (B) Treatment of feed *Chrysonilia crassa* in ileum (C) Control in coecum (D) Treatment of feed *Chrysonilia crassa* in coecum

Figure 2 shows the pattern of population dynamics LAB based on the analysis on the samples after every niche was compared between the control and treatment groups using *Msp* I restriction enzyme. In the ileum there was a difference in controls that was appear *Unidentified bacterium* (50 bp), while on treatment group appear *Lactobacillus* species *delbruekii* (180 bp) and *Lactobacillus* sp. (187 bp). The same species that can be found in the control and treatment

are *uncultured bacterium* (87 bp). While in the coecum there was a difference, that was in the controls group appear *Lactobacillus* species *delbruekii* (180 bp). The same species that can be found in the control and treatment are *uncultured bacterium* (87 bp) and *Lactobacillus* sp. (187 bp). It because the bacteria was the most dominant nature *unculturable* (not *cultured).* This was based on Jannah (2014) *phylotype* BAL 41% unidentified *(unidentified)* and 36% were identified as a bacterium that cannot be cultured *(unculturable).* According to Walter [19], *Lactobacillus* is a bacterium that is the dominant inhabitants of the gastrointestinal tract because it is easy to grow, has a high tolerance in the circumstance with or without oxygen (facultative anaerobes). *Lactobacillus* sp. in the *cecum* may help digest crude fiber by fermentation by *cellulolytic* bacteria [9].

The population dynamics of LAB are caused by many things. One of them is the quality of feed that affects the existence of bacterial community in the digestive tract. LAB requires nutrients such as carbohydrates, amino acids and vitamins to grow. The quality of raw materials, the nutrients content, mycotoxin contamination, and physical quality of the feed (particle size) can accelerate or retard the rate of movement in the digestive tract *(feed passage).* Enzymatic digestion is done by the intestinal wall, which produces protein-breaking enzymes (erepsin), fats (lipases), and carbohydrates (maltose and saccharase) [22]. Bran is one of ingredients which contains carbohydrates and easily digested.

Fermented *Chrysonilia Crassa* feed given as treatment, mold is believed to have a potential as probiotics but its use requires other types of microbes. Each type of probiotic has a special function, and more than one probiotic is mixed it can work synergistically. *Chrysonilia crassa* mold is specificallyable to produce the potential cellulase enzymes to degrade the lignocellulolytic material which causes the breaking of *oligosaccharides* into glucose and protease enzymes which is able to break down the protein compounds in the feed to be more easily absorbed so that it will increase the protein content in the biomass [21].

3.4. Relative abundance and diversity Lactic Acid Bacteria

The abundance of species will be more suitable determined by T-RFLP metagenom analysis, in addition the metagenomics T-RFLP analysis. In addition, it can detect the structure and composition of microbial communities in actual conditions as relative abundance can be compared between samples [4].

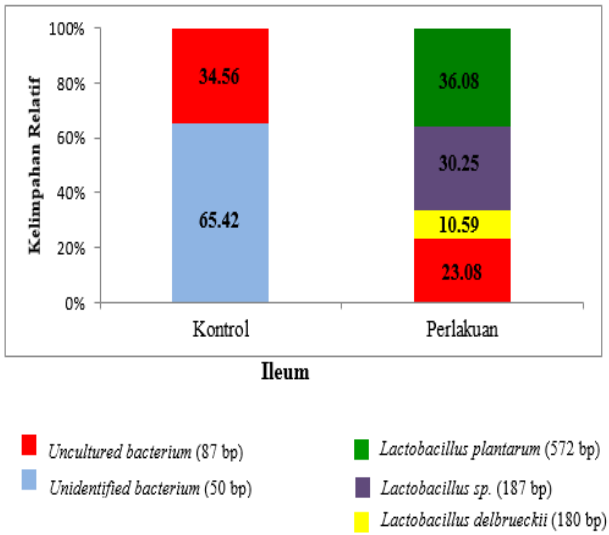

Figure 3. The relative abundance distribution of each T-RF size arising from the LAB community on ileal control and treatment

Figure 3 showed that the existence of *unidentified bacterium* (50 bp) is found in large quantities which is 65.42% in the hollow of the control ileum. This is due to the lack of reference from MiCA3 *database* to identify the results of all the pieces of BAL T-RF so that there is

unidentified pieces result. This is in accordance with Jannah [8] that 41% of LAB phylotype is unidentified and the other 36% were identified as unculturable bacterium. The treatment of ileum are found at most is *Lactobacillus plantarum* (572 bp) as much as 36.08%. *Lactobacillus plantarum* is a microbe found in *manyplant*-derived material containing *lignocellulose*. This is due to the treatment of bran fiber content is higher than commercial feed. This is in line with Li *et al.* [10] who stated that *Lactobacillus plantarum* is found mostly in the feed with a high fiber content. The growth of LAB can be influenced by several factors such as environmental conditions, the population of pathogenic bacteria, the type of feed, and the availability of substrate [11]. According to Yuwanta [22], the use of crude fabric which is 5-20% provides variety to the development, the number of bacteria, and the dynamics of cellulolytic bacteria in the small intestine, *cecum* and colon in poultry with different levels of crude fiber [22].

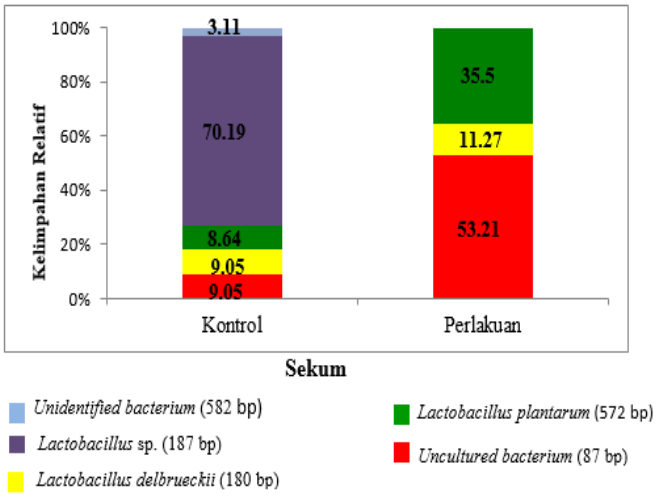

Figure 4. The relative abundance distribution of each T-RF size arising from the LAB community on the control and treatment of *cecum*.

Figure 4 shows that *Lactobacillus* sp. (187 bp) were found in abundant amounts of controls, which were found in the *cecum* as much as 70.19%. This increasing trend is due to the *cecum* as a very important place for the fermentation of foodstuffs, so there are found much *Lactobacillus* sp. in the metagenomic approach of the lumen content sample of the *cecum*. *Lactobacillus* sp. can decrease the pH, so the atmosphere in the *cecum* becomes acidic that is able to ferment food that resulted in the decrease in the number and activity of gram negative bacteria [15].

The relative abundance shows the LAB community in the *ileum* and *cecum* of broilers before and after being treated with fermented *Chrysonilia crassa* feed. The LAB community which were detected on metagenom approach were *Lactobacillus delbrueckii* (180 bp), *Lactobacillus* sp. (187 bp), *Lactobacillus plantarum* (572 bp), *uncultured bacterium* (87 bp, 582 bp) and *unidentified bacterium* (50 *bp).*

The analysis of phylotype, diversity index, eveness index, and similarity index on ileum and coecum broiler chickens were conducted based on *Hae*III and *Msp*I profile data. The diversity index can be used to express the relationship of phylotype abundance in a community. In this case the diversity is seen from the number of phylotype s, abundance and resemblance of the community by using restriction enzyme data profiles used on the basis of the T-RF results of the BAL community [8].

Table 1. Comparison of Total phylotype, Diversity, and Evenness of BAL Community T-RFLP Analysis Results

	Ileum		Coecum	
	Control	Treatment	Control	Treatment
The number phylotype (S)	3.5	4	4.5	2
Pielous Eveness index (E)	0.67 0.48	0.70 0.69		
Shannon-Wiener diversity Index (H')	0.84 0.66	1.05 0.48		

Most of the data obtained showed that the number phylotype of in a community with restriction enzyme Msp1 showed more phylotype than *Hae*III. In comparison, the cecum community on control using the restriction enzyme *Hae*III found 4 phylotype, whereas using the restriction enzyme *Msp*1 found 5 phylotype. By the difference, in this study using the average data obtained from two restriction enzymes used. Shannon-Wiener's (H ') diversity index in the LAB community on control and treatment.

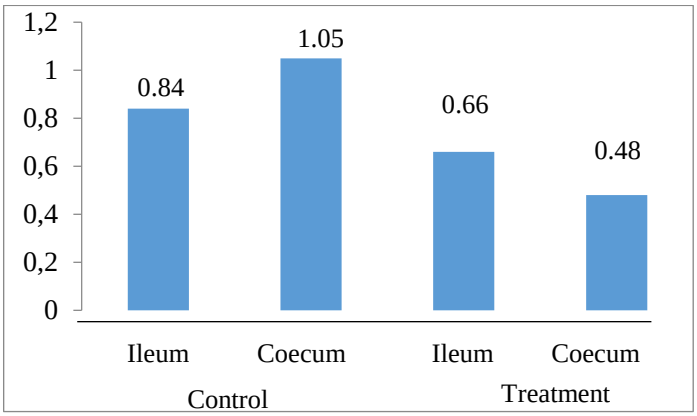

Figure 5. The Shannon-Wiener Diversity Index (H ') of the LAB community on control and treatment

Figure 5 shows the Shannon-Wiener diversity index BAL between two hollows of the digestive tract of broilers which are in the ileum and *cecum*. The control diversity index in ileum is (0.84) while in *cecum* is (1.05). This shows that the control of the BAL community in the *cecum* tends to be more diverse than the community in the ileum. This is based on the Shannon index value between 1.0 and 6.0 (Stiling, 1996). The treatment with feeding of fermented *Chrysonilia crassa* in the ileum showed the diversity index of 0.66, while in the *cecum* 0:48.

This shows that in the treatment of LAB communities in ileum has a low diversity. The calculation in every hollows shows that the value of the diversity index *(H ')* of LAB included in the category of low to high, as the categories, namely, H'> 3.0 indicates a very high diversity, 1.6 <H ' <3.0 indicates high diversity, 1.0 <H '<1.5 indicates moderate diversity, and H' <1.0 indicates a low diversity. The values of diversity index obtained ranged from 0.48 to 1.05. Based on the results of the diversity index, it indicates the moderate category H '= 1.05 is found in the control *cecum* hollows. This illustrates that the treatment of commercial feeding causing the diversity of LAB communities is higher in *cecum* hollows than in ileum, because the species of LAB phylotype more widely available in *cecum*.

The stability of a species is also influenced by the level of evenness, the higher the value of H ', the more stable diversity of species in the community. In contrast, the lower the H' value, the lower of species stability level of species diversity in the community [14].

Enzyme	Control-Treatment		Control	Treatment
	Ileum-Ileum	Coecum-Coecum	Ileum-Coecum	Ileum-Coecum
*Hae*III	0	0	0.44	0
*Msp*1	0	0.5	0	0.29
Average	0	0.25	0.22	0.15

The *Sorensen pairwise* similarity is a value that indicates phylotype similarities in the two communities. The value of indicates that two communities are completely different, while a value of 1 means that two communities are identical. In this study, table 1 shows the highest similarity index (0.25) is between the coecum communities contained in the control and treatment. It means the highest similarity of LAB composition of the gastrointestinal tract in broilers is between the coecum communities while in 2 different communities but in the same environment (control) have similarity value (0.22).

This means that the two communities on the controls are different because they are close to 0. This is based on Stiling [16] who stated that the equality index values are 0-1, which means 0 there is no overlapping phylotype in the community, the value of 1 means the exact same phylotype in both communities [16]. The high similarity in coecum is assumed that LAB viability is high in cecum whereas high similarity in feed effect (control) is due to the absence of changes in the composition of the content in commercial feed due to enzymatic process as in fermentation feed which can affect the LAB community on ileum and coecum.

4. Conclusion

Based on T-RFLP analysis by looking at T-RF profile, there were differences in community diversity of *Lactic Acid Bacteria* (LAB) in the ileum and cecum of broilers fed with the fermented *Chrysonilia crassa.* Feeding treatment with fermented *Chrysonilia crassa* (treatment) reduced the level of LAB diversity compared to the provision of commercial feed (control).

5. Optional

Based of the conclusion of the research to apply to the chicken, feeding fermentation *Chrysonilia crassa* need to note the dosage used and application of fermented *Chrysonilia crassa* is not effective to see the level of chicken health

Acknowledgments

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