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# Analysis of Rumen Microbial Population of Cattle Given Silage and Probiotics Using Terminal Restriction Fragment Length Polymorphism

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Rumen ecology is an important observation in evaluating the effectivity of silage and probiotic additives relating to their roles in cattle productivity. The objective of this study was to examine the effects of silage and probiotics on ruminal ecosystems *in vivo* using a molecular approach. Terminal-restriction fragment-length-polymorphism (T-RFLP) analysis was used to detect changes of ecological communities based on 16S-ribosomal deoxyribonucleic acid (16S-rDNA). Two rumen canulated PO cattle were fed several diets *i.e.*; (R0) basal diet dry matter basis (*Pennisetum purpureum* 70% and commercial concentrate 30%), (R1) silage (basal diet fermented using *Lactobacillus plantarum* BTCC570), (R2) silage + probiotics (*L. plantarium* Str BTCC531), (R3) Basal diet + probiotics (*L. plantarium* Str BTCC531). Digesta samples were collected 3 h after feeding for pH and T-RFLP analysis. T-RFLP analysis was performed using the 16S-rDNA amplified from each sample. The lengths of the terminal restriction fragments were analysed after digestion with *Hha*I, *Hae*III and *Msp*I. Results showed the effectivenes of silage and probiotics, given together, on the index of Smith and Wilson evenness applied to T-RFLP ecology data (Evar) with 0.89±0.04 being the highest. The diversity of rumen microorganisms is influenced by individual differences of each animal. T-RFLP analysis has a potency to be used for comparisons of complex bacterial communities, especially to detect changes in community structure in response to different variables and to show rumen bacteria diversity in the rumen.

Keywords: silage, probiotics, rumen ecology, Evar, T-RFLP, 16S-rDN

The rumen is a complex ecosystem in which animal feed is consumed by ruminants and digested by an active and diverse microbiotal population. Most of the microorganisms aid digestion, but others may potentially cause pathogenesis. The implication of microbiota in the rumen for nutrition and feed conversion of the animals merits greater attention. Microbiota in the rumen consists of mainly bacteria, followed by protozoa and fungi. Information on number of cultured bacteria is around $10^{10}$, protozoa $10^{6}$ and fungi $10^{4}$ cfu $mL^{-1}$ rumen content (Hungate 1966).

The number and composition of rumen microbiota were influenced by the composition of the feed consumed by the animals. Naturally, the feed contains roughage and concentrate, but it may also contains feed additives such as directly fed microorganisms or probiotics. Several microorganisms have been widely selected and used as probiotics. Lactic acid bacteria (LAB) are considered 'Generally Recognized as Safe' (GRAS) and some species have been selected as probiotics. LAB present in young animals are about $10^{6}$ cfu $mL^{-1}$ and the number decreased when the animals changed the type of feed being consumed. The presence of LAB in the rumen is related to, firstly, acidosis caused by excess concentrate consumption and secondly, probiotic properties (Stewart 1992). Probiotics have been reported to give a positive effect to the rumen environment, prevent the gastrointestinal tract from infection, and therefore improve animal productivity (Fuller 1992).

Silage is a fermented ruminant feed of high moisture content. The process of fermentation is called ensilage and depends on the activity of LAB to convert water-soluble-carbohydrates into organic acids, mainly lactic acid. LAB are still alive in silage, and the consumption of silage by animals gives opportunity for LAB to enter the rumen (Weinberg *et al*. 2004).

The detection of rumen bacterial population by simple culture methods may give unreliable results, since many of the bacteria present in the rumen are not able to be cultured by available methods. The molecular tool commonly used for examining microbial communities is the Small-Subunit-ribosomal-Deoxyribo-Nucleic-Acid (SSU-rDNA). Terminal-restriction fragment-length-polymorphism (T-RFLP) analysis is one of the procedures that can be used to track spatial and temporal changes in SSU-rDNA's from microbial communities (Liu 1997). Among the available procedures, T-RLFP is suitable to give a rapid comparison of complex bacterial communities. T-RFLP has been used for detecting the bacteria in the rumen of unfaunated and faunated cattle. T-RFLP patterns showed that the absence of protozoa in the rumen changes the composition of fecal bacteria (Ozutsumi *et al*. 2008). Probiotic strains ingested by humans were able to survive in the intestine and tracked in the feces as indicated by the corresponding T-RFs (Bakir *et al*. 2008).

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The aim of the present study is to analyze the effect of the administration of LAB from silage and probiotics on the rumen population of cattle using T-RLFP.

## MATERIALS AND METHODS

**Bacterial Strains and Media**. *Lactobacillus plantarum* BTCC 570 and *L. plantarum* BTCC 531 were from the Biotechnology Culture Collection (BTCC)-LIPI. *L. plantarum* BTCC 570 was used as noculants for silage fermentation and *L. plantarum* BTCC 531 was used as a probiotic. Medium deMan rogosa sharpe (MRS) (de Man *et al*. 1960) was used throughout the study.

**Silage.** Elephant grass (*Pennisetum purpureum*) was preserved as silage. A commercial concentrate 10% (w/w) was mixed during the preparation of silage. The nutrient composition of the concentrate was crude protein 14%, fat 4%, crude fiber 8%, digestible protein 11%, total digestible nutrient (TDN) 68% and ash 10%. *L. plantarum* BTCC 570 ($10^{10}$ cfu $mL^{-1}$) 0.1% was used as a inoculant by spraying and mixed thoroughly with *P. purpureum* and concentrate. Plastic bags were used as a silo for the fermentation of silage. Incubation was for 30 days. The pH and population of LAB was determined after fermentation.

**Probiotics.** *L. plantarum* BTCC 531 was cultivated in MRS medium for 18 h at 30ºC and harvested by centrifugation at 16 770 x g. The resulting pellet was mixed in 10% w/v skimmed milk solution, then freeze dried overnight. The resultant probiotics powder was put in soft capsules of 400 mg.

**Animals and Sample Collection**. Two rumen fistulated 'Peranakan Ongole' (PO) cattle (Cattle A and B) were reared at the animal house of the Research Center for Biotechnology in Cibinong. They were given several diets, *i.e*. a basal diet consisting of 70% dry matter fresh elephant grass (*P. purpureum*) and 30% commercial concentrate (R0), silage of *P. purpureum* (R1), R1+ 1 capsule of probiotics (R2) and R0 + 1 capsule of probiotics (R3). The amount of dietary material given was calculated based on the body weight of each animal. The diets were given 2 X a day at 08.00 and 13.00 hrs, while water are available *ad libitum*. Adaptation and treatment periods for each diet were 10 and 14 days, respectively. During the treatment period, samples of rumen content was collected on days 5, 10, and 14. Samples of rumen content were collected 3 h after the morning feeding through the fistula, and squeezed using a double layer of cheese cloth. Samples were kept in 20 mL sterile corning tubes and stored at -20ºC until analysis.

**Extraction of DNA**. 10 g of each rumen sample was squeezed using a double layer of cheese cloth and thoroughly mixed. Squeezed rumen samples (200 ìL) were washed 2 X using phosphate buffer saline (800 ìL) and centrifugation at 14 534 x g for 2 min. The pellet was used for DNA extraction. Bacterial DNA was extracted using the Dneasy Blood and Tissue DNA extraction kit (Qiagen, Japan) according to the manufacturer's instructions. The purity of DNA was measured using Gene Quant pro (Amersham Biosciences-England).

**PCR Amplification.** Two universal primers 46F (5'GCYTAACACATGCAAGTCGA-3') and 1080R (5'-CCCAACATCTCACGAC) were used in PCR to amplify the 16S-rDNA coding region. The 46F primer was labeled with 6-carboxyfluorescein (6-FAM, Sigma Tokyo, Japan). PCR amplification was performed in a total volume of 50 ì L containing 1 ìg of DNA, concentration of primer 6FAM-46F; 0.4 ì M, primer 1080R; 0.4 ì M and Premix Taq® 25 ìL (Takara Bio Inc. Otsu, Japan). The PCR was conducted using a thermocycler Biometra®Tgradient (Thermoblock-Germany). The PCR conditions were based on the method of Dinoto *et al*. (2006b) with the following modifications: heating 94ºC for 2 min, followed by 30 cycles consisting of 94ºC for 2 min, 48.5ºC for 1 min and 72ºC for 1 min and a final extension period of 72ºC for 10 min. The PCR product was purified by SUPREC PCR (Takara Bio Inc. Otsu Japan). Purified PCR products from individual DNA extractions from 3 collection times (day 5, 10, and 14) from each diet were combined and digested with restriction enzymes *i.e. Hae*III (50 U ì $L^{-1}$), *Hha*I (10 U ì $L^{-1}$) and *Msp*I (50 U ì $L^{-1}$) (Takara Bio Inc.) at 37ºC for 24 h. The reaction was terminated by placing the microtubes with individual samples in a water bath at 65ºC for 20 min, and then put on ice immediately. The restriction digest products were subjected to ethanol precipitation and vacuum drying.

**T-RFLP Analysis.** The lengths of the terminal restriction fragments (T-RFs) were determined with standard sized marker 500 Lz using ABI PRISM 3100 genetic analyzer at Atmajaya University and Genescan Analysis 3.7 software (Applied Biosystem). The major T-Rfs were identified by computer simulation, which was performed using 16S-rRNA gene sequences registered with the Ribosomal Database Project (RDP) II (http://rdp8.cme.msu.edu/html/TAP-Java2.html). The dendrogram analysis was based on the similarity coefficient for the objective interpretation of the difference T-RFs patterns. Dendrogram-type was established using the unweighted pair-group method with arithmetic mean (UPGMA) (Blackwood *et al*. 2003).

**Analysis of the Data.** The data obtained from the T-RFLP analyses were normalized as described by Sait *et al.* (2003). T-RFs peak areas with total areas less than 5% threshold value were excluded. A Sorensen's pairwise similarity coefficient, *C*s, was calculated for each pair of T-RFLP profiles within a complete edited data set as follows:

$$Cs = 2j/(a+b)$$

where *j* is the number of T-RFs with relative areas of greater than zero common to the two profiles being compared within an edited data set, and *a* and *b* are the number of T-RFs with a relative area of greater than zero in each of the two profiles. Diversity of rumen bacteria populations were base on method diversity index Smith and Wilson *evenness* (E*var*) described by Blackwood *et al*. (2007) given below:

$$Evar = 1 - \frac{2}{\pi} \arctan\left\{\left[\sum_{i=1}^{s} \ln (pi) - \sum_{i=1}^{s} \ln (pi)/s\right]^2/S\right\}$$

## RESULTS

**Silage and Probiotics.** Silage has been created with the following characteristics; soft texture, not moldy, sour aroma typical of lactic acid, pH 3.99, dry matter 32.78% and a total population of LAB around 1.1 x $10^6$ cfu $g^{-1}$ silage. Probiotic broth has been created with a population density of 7.5 x $10^{11}$ cfu $mL^{-1}$ with a value of $A_{600}$ of 1.7. Probiotic is further made into probiotic powder using freeze-drying to produce a population density of 1.67 x $10^{13}$ cfu $g^{-1}$.

**Extracted and Amplificated DNA of Rumen Bacteria.** DNA concentration of extracted cattle rumen contents with a total volume of 100 ìL in the range of 31.71-128.40 ng ì $L^{-1}$ and a DNA vs RNA purity was measured by $\Delta A_{260/230nm}$ of 1.05-1.26 and DNA vs protein $\Delta A_{260/280nm}$ of 0.98-1.20. PCR amplification of 50 ìL reaction mixture has been successfully performed from all treatments with the resulting size of 1000-1100 bp, in accordance with if the primer in use is 6FAM-46F and 1080R (Fig 1). Amplicon concentration ranging between 53.00-116.40 ng ì $L^{-1}$ with DNA vs RNA purity on $\Delta A_{260/230nm}$ and protein vs DNA on $\Delta A_{260/280nm}$ each of 1.82-2.57 and 1.17-1.92.

**Similarity Coefficient ($C_s$).** Normalization is performed on each sample using a method based on the percentage of area that is the comparison between the relative peak area detected by the total peak area of all communities of enzyme used multiplied by 100%. A percentage greater than 5% of the population was considered to be stable and to represent the most factual bacterial population in the community (Sait *et al*. 2003). Table 1 shows that the treatments R2 with R3 and R0 with R3, have the highest Cs values. Treatment R0 and R2 show the lows L-value for Cs, so the bacterial population in the R2 has a difference of treatment which is far with the R0 treatment. A close relationships of organisms in the rumen can be seen from the dendrogram which uses clustalX2 through application of the T-RF pattern based on UPGMA. In principle clustering by UPGMA is almost the same as the value of the Cs (Fig 2).

Tabel 1 Similarity coefficient ($C_s$)

| Treatment | Animal | | Average |
|---|---|---|---|
| | A | B | |
| R2 - R3 | 0.44 | 0.63 | 0.53 ± 0.13 |
| R0 - R3 | 0.29 | 0.75 | 0.52 ± 0.33 |
| R0 - R1 | 0.27 | 0.63 | 0.45 ± 0.25 |
| R1 - R3 | 0.24 | 0.65 | 0.44 ± 0.29 |
| R1 - R2 | 0.32 | 0.57 | 0.44 ± 0.29 |
| R0 - R2 | 0.26 | 0.60 | 0.43 ± 0.24 |

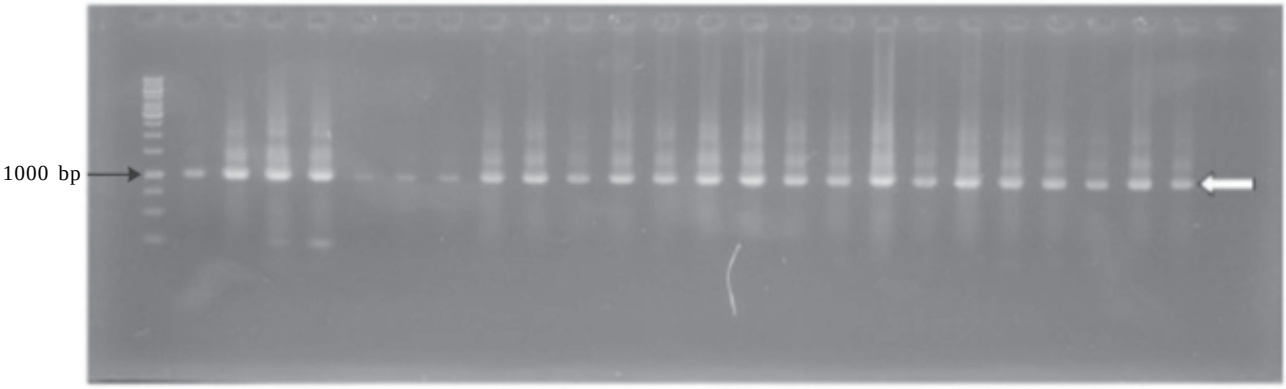


Fig 1 Amplified DNA of rumen bacteria from all treatments.

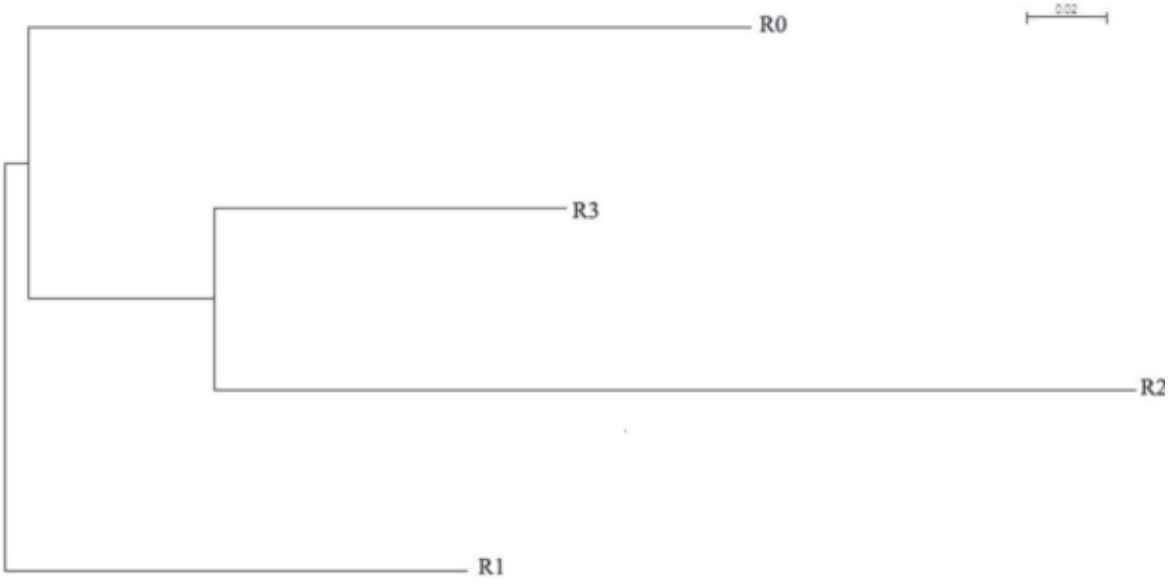


Fig 2 The dendrogram illustrates the relationship of the T-RFLP pattern from rumen bacteria by *Hae*III, *Hha*I, and *Msp*I restriction enzymes base on UPGMA clustering.

Tabel 2 Diversity indexs Smith and Wilson *evenness* (E*var*)

| Treatment | Animal | | Average |
|---|---|---|---|
| | A | B | |
| Silage + Probiotic (R2) | 0.92 | 0.86 | 0.89 ±0.04 |
| Basal diet (R0) | 0.72 | 0.81 | 0.77 ±0.07 |
| Silage (R1) | 0.73 | 0.69 | 0.71 ±0.03 |
| Basal diet + Probiotic (R3) | 0.46 | 0.85 | 0.65 ±0.27 |

**Diversity Indexs Smith and Wilson *Evenness* (E*var*).** Ecological diversity of bacterial communities from a data scaning can be seen with the index diversity use of Smith and Wilson *evenness* (E*var*). A third enzyme was used in T-RFLP analysis for strengthening the data obtained by observation. Given R0, R1, R2, R3 and the second cattle PO showed the diversity of rumen bacteria that have a sufficient interest to the area with a population of fluorescence intensity that was around 28, 29, 26, and 25 T-RFs of all enzyme is used in this research. R0 is quite diverse with the E*var* value higher than those of R1 and R3 (Table 2). Interestingly, R2 as a treatment of silage and probiotic combination showed a synergistic effect that increases the E*var* value to be highest amongst all treatments.

**Closeness of T-RF (bp) with the RDPII Organisms.** To know the names of the population of rumen bacteria from closeness and diversity of each treatment, the size T-RFs was next compared with genebank database on the Ribosomal Data Project (RDP) II on the homepage http://rdp8.cme.msu.edu/html/TAP-java2.html. Data in Table 3 show the closeness of T-RFs observations with prediction organisms from databases on the three restriction enzyme used. The genescan ABI Prism 3100 results are shown in the form of on electropherogram for all treatment of the two cattle which were digested with *Hae*III enzyme (Fig 3).

## DISCUSSION

The process of PCR amplification using label primer at the 5' end of the with 6-carboxylfluorescence-amplified-specific-regions of bacterial genes 16S-rDNA content of total community DNA was succesful. The automatic genetic analyzer only read fragments of nucleic the flourescence at the 5' end of the absorption by the sensor when loaded at the capillary (Liu *et al*. 1997; Kaplan *et al*. 2001).

Because the amount of DNA loaded on the capillary cannot be accurately controlled, the sum of all T-RF peak areas in a pattern (total peak area) varied between T-RF patterns. To compensate for this variation, it was necessary to normalize peak detection thresholds and totals of peak areas (Kaplan *et al*. 2001). Osborne *et al*. (2006) states that to minimize variation, bias, and to ensure the size of T-RF which was found was genuine one should be normalize performance on certain thresholds.

The high value of Cs for the treatment had a structure similar to the organisms in the rumen. While the small value of Cs showed differences between organisms that were found in the rumen. Closeness of organisms in the rumen can be seen from the dendrogram also use clustalX2 through application of the T-RF pattern based on UPGMA (Fig 2). The treatment R2 is able to alter the community of previously dominant to be a population in decline and creation of new ones. The new population shows the role of ecology in the rumen, so that the old population will not necessarily fit with the new environment.

Diversity of rumen microorganisms depends on feed, the condition of the cattle, and sinergism with other microbes. Equality in this equation, as compared to the other indexes in the analysis, showed that a more ecological approach was closest to reality and to have a high correlation (Blackwood *et al*. 2007). In our study, an increased diversity

Table 3 Closeness of T-RFs lengths (bp) with organism on Ribosomal Database Project II

| Organism | T-RFs length (bp) | |
|---|---|---|
| | Predicted (HaeIII,HhaI,MspI) | Observed (HaeIII,HhaI,MspI) |
| Bifidobacterium angulatum ATCC27535(T) | 46, 335, 43 | 46,338, ND |
| Clone EH-7 | 152, 59, NA | 152, 59,55 |
| str.rj5 | 152, 159, 93 | 152, 161, 95 |
| Clone WCHBI-82 | 161, 331, 250 | 161, 331, 256 |
| Clone12-102 | 181, 64, 59 | 181, 65, 57 |
| Eubacterium cellulosolvens ATCC43171(T) | 201, 152, 184 | 201, 150, 184 |
| Syntrophomonas saporans | 201, 341, 259 | 201, 339, 258 |
| Clone vc2.1 Bac43 | 201, 23, 259 | 201, ND, 258 |
| Eubacterium ptautii DSM 4000(T) | 201, 350, 260 | 201, 349, 258 |
| Clostridium fusiformisCm973 | 201, 1052,184 | 201, ND, 184 |
| Arcobacter cryaer ophilus CCUG17801 | 224, 64, 436 | 223, 67, 436 |
| Arcobacter butzleri CCUG10373 /(CloneT31/CloneT55/Clone T95) | | |
| Ruminococcus productus ATCC27340/ Clostridium sp. strain DR6A | 233, 150, 182 | 233, 150, 184 |
| Ruminococcus hansenii ATCC 27752(T) | 235, 151, 259 | 235, 150, 258 |
| Cytophaga fucicola NN015860/sw17 | 242, 56, 51 | 243, 59, 51 |
| Epulopiscium sp. strain morphotype A2/ Epulopiscium fishelsoni Red. Sea A2 | 247, 334, 244 | 247, 331, 241 |
| Bifidobacterium longum ATCC15707(T) | 247, 334, 96 | 247, 331, 94 |
| Metabacterium polyspora | 251, 517, 248 | 251, ND, 248 |
| Clostridium purinolyticum ATCC33906(T) | 260, 160, 128 | 260, 161, 123 |

index in silage and probiotic treatment is related to the beneficial effects on cattle productivity, in which much more propionic acid is produced in rumen (Ridwan unpublished data). Although, the presence of *L. plantarum* (silage inoculant) and *Leuconostoc* sp. (probiotic) could not be detected in the rumen five days after consumption, the data of increased bacterial diversity and rumen metabolic changes (data not shown) indicate indirect roles of those microorganisms in rumen ecology. New microorganisms might exist in low numbers in a population at early on after additives were consumed and disappear several days after due to the complex environment in the rumen. However, those microorganisms which are able to change the microbial diversity in rumen and finally support the production beneficial metabolites for host most likely exist.

Closeness of restriction sites of microorganisms that are contained in the database tolerate an accuracy between T-RF data observations with T-RF predictions of more than 3

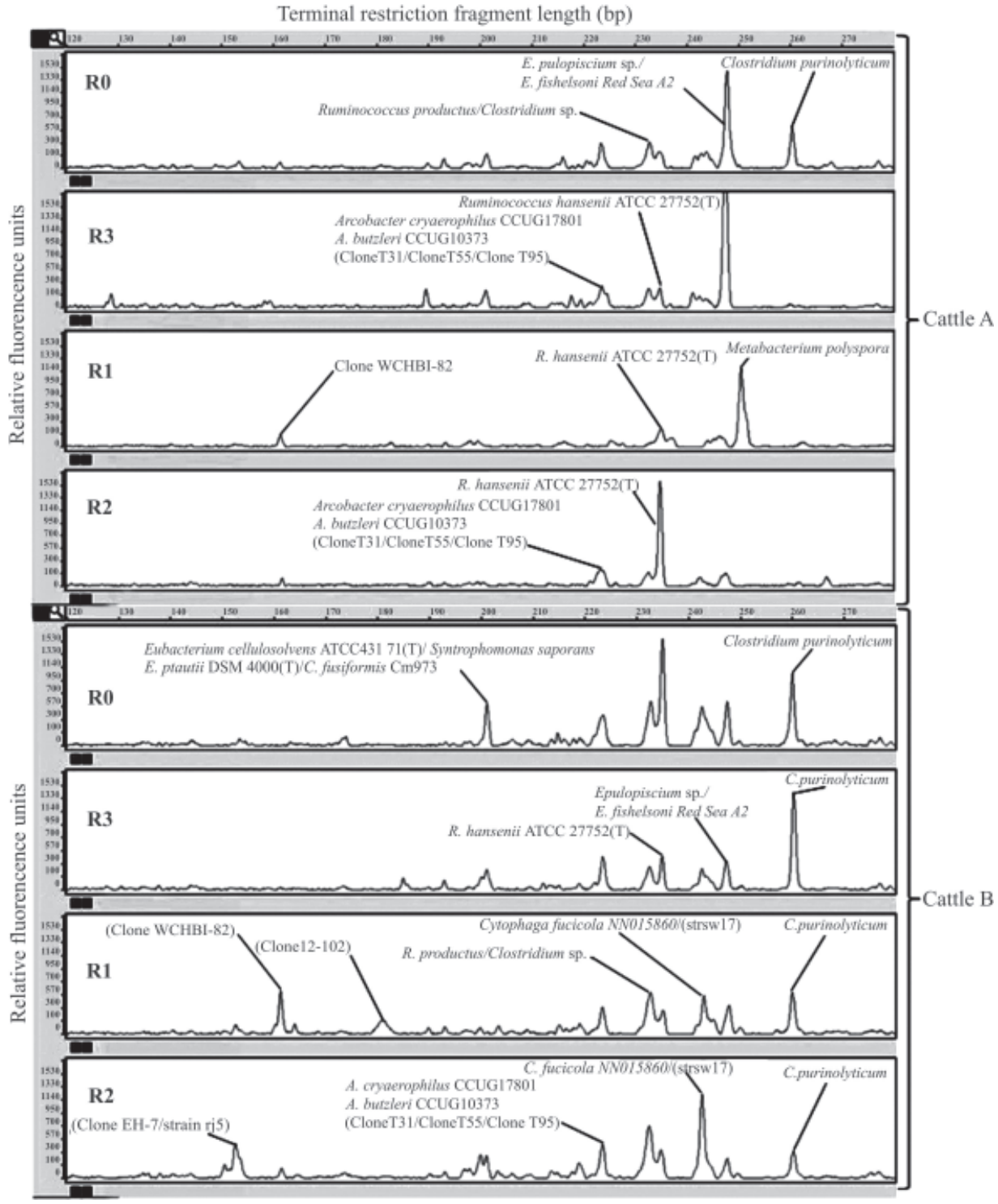


Fig 3 T-RFLP patterns of 16S rDNA's from rumen samples of two peranakan ongole breed cattle from a basal diet (R0), probiotic (R3), silage (R1), and silage + probiotic (R2) treatment generated after digestion with *Hae*III restriction enzyme. 16S rDNAs were amplified with universal primers FAM-46F and 1080R.

bp (Sakamoto *et al*. 2004). There was a tendency of a decline in the population of *Clostridium purinolyticum*, which was originally found in the R0 treatment, when cattle were fed with silage (R1) or silage plus probiotic (R2) (Fig 3.). The population of *Epulopiscium* sp. Morphotype A2/*E. fishelsoni* str Red.SeaA2/*Bifidobacterium longum* ATCC15707 (T) in both experimental animals decreased when treated with R1, as well as with R3. *Metabacterium polyspora* appeared in rumen of cattle A when R1 was applied as feed.

The population of *Ruminococcus productus* ATCC 27340/*Clostridium* sp. strain DR6A on the cattle A decreased dramatically when this animal received R1, but their was an increase in the population of this organism in the R3 treatment. This is similar with that of animal B, where R2 treatment showed indications of an increased population *R. productus* ATCC 27340/*Clostridium* sp. strain DR6A Str. Thus, there are indications that a given probiotic encourages rumen stimulation based on the presence of bacteria that previously did not exist, or were scarce. This also occured in both cattle A and cattle B, with a decrease in the population of *Cytophaga fucicola* str NN015860/str sw17. Taken together, treatments of all feed consumed by the PO cattle clearly shows the diversity of rumen ecology (Table 3). A decline in the population of dominant bacteria in the rumen after feed-treatment stimulated the growth of other bacteria in proportions detectable by T-RFLP analysis, although this new population is still relatively low.

CloneT31/CloneT55/CloneT95/*Arcobacter cryaerophilus* CCUG17801/*A. butzleri* CCUG10373 in the rumen of cattle A treated with R1 eliminated the dominant bacteria in the population such as *M. polyspora*. *Metabacterium polyspora* is actually a gram-positive anaerobic bacterium and was originally found in the digestive tract of pigs (Esther *et al*. 1998). Clone WCHBI-82-102 and Clone12 appeared significantly after decreasing *R. hansenii* ATCC 27752 (T) and *C. purinolyticum* ATCC33906 (T). *Clostridium purinolyticum* is well known as gram negative bacterium that is capable of degrading protein. This bacterium grows optimally at pH 6.5-9.0 under anaerobic conditions (Durre *et al*. 1981).

This study reflects the dynamics of bacterial populations towards feed modulations. The succession of microbe close to Clone WCHBI-82/Clone12-102, *Epulopiscium* sp. strain *morphotype* A2/*E. fishelsoni* Red Sea A2/*B. longum* ATCC15707 (T), and *C. purinolyticum* ATCC33906 (T) was noted after reduction of levels of this organism which was followed by an increase in the population of clone EH-7/ str.rj5 and *C. fucicola* NN015860, str sw17. In a previous study, it was reported that the pattern of decrease and increase in the population of certain bacteria correlates with the improvement of the immune response under stress conditions, the stimulation of microbial growth in the rumen and the stabilization of rumen acidity (Krehbiel *et al*. 2003).

Almost all organisms, including anaerobic bacteria, can grow at a temperature of 35-43$^{o}$C, derived from the digestive tract of cattle, other ruminants and nonruminants, except for Cytophaga. This organism is an aerobic bacteria, isolated from sea water, and can hydrolyze cellulose substrate (Johansen *et al*. 1999).

As shown in Table 3, *B. angulatum* has the closest T-RF observation value when compared the to T-RF prediction values stored in the database. Since *B. angulatum* was reported as an original organism of the human digestive tract (Dinoto *et al*. 2006a), the existence of this organism needs to be clarified in the future using a PCR method with a specific primer, or by using a clone library approach. The diversity of rumen microorganisms is influenced by the individual differences of each animal. This can be seen with the appearance of some of the population that occurred in cattle A, but not in cattle B.

In conclusion, this study clearly shows that the feed additives used influence the diversity of the microbial population in the rumen as revealed by T-RFLP analysis. The highest diversity (E*var* Smith and Wilson *evenness* = 0.89±0.04) of rumen microbial community, which was found when cattle received silage and probiotics simultaneously. This phenomenon reflects the synergistic effects of a change of the microbial population and of metabolites in the rumen.

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