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PREFACE

In our edited and opened access journal, as well as free publishment we proudly present articles from Animal and Veterinary Sciences. This edition, volume 23 no 1 March 2018 comes from Breeding and Genetic, Feed and Nutrition Technology, and Veterinary Science, those are: "Genetic diversity and structure of native Egyptian cattle populations and French-Egyptian cross via DNA-microsatellite", "Plant extract activities as antioxidant and antibiofilm against chicken gut bacteria"; "Bioactive substances of some herbals and their effectiveness as antioxidant, antibacteria and antifungi"; "Substitution of mulberry leaf meal on feed intake, body weight and carcass characteristics of Tigray highland lambs"; "Effect of supplementation of BS4-enzyme levels in rice-bran based rations on performance of growing PMp broiler duck" and "Epidemiology of traumatic myiasis due to *Chrysomya bezziana* in Indonesia".

Hopefully these articles would offer any benefit to readers and the end-users of technological innovation. We extent high appreciation to all authors and peer reviewers those contribute in this edition that would make this journal published. We hope other authors will contribute in the future.

Chief Editor;

Bogor, March 2018

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Genetic Diversity and Structure of Native Egyptian Cattle Populations and French-Egyptian Cross via DNA-Microsatellite

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ABSTRAK

Faid-Allah E, Ghoneim E, Elbetagy AR, El-Dabour M. 2018. Perbedaan dan struktur genetic populasi sapi Mesir lokal dan persilangan sapi Perancis-Mesir melalui penandaan DNA Mikrosatelit. JITV 23(1): 1-10. DOI: http://dx.doi.org/10.14334/jitv.v23i1.1647

Penelitian ini bertujuan untuk mempelajari perbedaan dan struktur genetik populasi sapi Mesir lokal Baladi jenis Saidi yang berasal dari Mesir bagian selatan, Menufi dari Mid-Delta dan persilangan F1 keduanya dengan jenis Taretaise Perancis melalui penandaan DNA Microsatelit. Sebanyak 97 ekor sapi yang tidak berhubungan dianalisis secara genotip menggunakan 8 primer SSR (ETH10, ETH225, BM1818, BM1824, BM2113, SPS115, TGLA53 dan TGLA126). Seluruh SSR yang digunakan mernunjukkan sifat polimorfik. Jumlah tertinggi dan terendah alel adalah 16 pada loci TGLA53 dan 6 pada loci SPS115. Persilangan Baladi-Tarentaise memiliki jumlah alel terbanyak secara keseluruhan. Nilai informasi polimorfik (PIC) pada 7 loci lebih tinggi dibandingkan dengan loci 0,5 yang mengindikasikan variasi alel yang tinggi pada penanda yang digunakan. Nilai perkiraan PIC bertutur-turut adalah 0.898; 0,866 dan 0.873 untuk loci TGLA53 dari genotipikasi sapi Saidi, Menufi dan Bal-Tar. Nilai heterozigositas yang diamati lebih rendah dibandingkan dengan nilai perkiraannya pada populasi lokal diikuti dengan nilai F_{is} positif dan deviasi yang signifikan dari HWE yang mengindikasikan kecenderungan perkawinan sedarah di dalam populasi lokal tersebut. Analisis struktur mengindikasikan bahwa tiga genetik tetua. Populasi sapi lokal tersebut membagi dua asal tetua utama dengan persentase yang hampir seimbang, sementara itu persilangan Bal-Tar memiliki asal tetua ketiga. Ketiga populasi menunjukkan persentase campuran yang rendah. Populasi sapi Mediterrania yang dipelajari berasal dari Mesir dan Perancis sepertinya telah dibedakan satu sama lain dengan sedikit pertukaran genetik antar yang populasi yang terisolasi secara geograpis sehingga sapi lokal memiliki kemiripan yang tinggi.

Kata Kunci: Sapi, Keanekaragaman Genetik, Mikrosatelit DNA

ABSTRACT

Faid-Allah E, Ghoneim E, Elbetagy AR, El-Dabour M. 2018. Genetic diversity and structure of native Egyptian cattle populations and French-Egyptian Cross via DNA-microsatellite. JITV 23(1): 1-10. DOI: http://dx.doi.org/10.14334/jitv.v23i1.1647

This study investigates genetic diversity and structure of native Egyptian cattle populations, called Baladi, as Saidi from Southern Egypt, Menufi from Mid-Delta and their F1 crosses with the French Tarentaise breed using DNA Microsatellite markers. A total of unrelated 97 individuals were genotyped utilizing eight SSR primers (ETH10, ETH225, BM1818, BM1824, BM2113, SPS115, TGLA53 and TGLA126). All utilized SSR were found to be polymorphic. The highest and lowest numbers of alleles detected were 16 and 6 at TGLA53 and SPS115 loci, respectively. Baladi-Tarentaise crosses (Bal-Tar) had the highest number of alleles over all. The PIC values of 7 loci were higher than 0.5, indicating high allelic variation of utilized markers. Estimated PIC values were up to 0.898, 0.866 and 0.873 for TGLA53 genotyped in Saidi, Menufi and Bal-Tar, respectively. H_{obs} values were lower than the expected ones in the native populations accompanied with positive values for F_{is} and significant deviation from HWE indicating inbreeding trend in native populations. Structure analysis indicated three ancestral genetic backgrounds. The native populations share two main backgrounds in almost equal percentages, while the Bal-Tar had the third one. The three populations showed low percentage of admixture. The studied Mediterranean cattle populations that belong to Egypt and France seem to have differentiated from each other with only little genetic exchange between the geographically isolated populations so local cattle is very similar.

Key Words: Cattle, Genetic Diversity, DNA Microsatellite

INTRODUCTION

Cattle are an important source of meat and milk in Egypt. They are distributed all over the country, with higher density in the Nile valley and delta and usually found in small holdings along with buffaloes. The Egyptian native cattle, called Baladi, had four breeds, being Domiati, Mariuti, Menufi, and Saidi (MoDAD 2004), that are defined mainly according to geographical distribution and minor phenotypic variations. Cattle breeds in Egypt lack molecular characterization required for establishing adequate

utilization of their genetic variation for the improvement of cattle production under the challenging local conditions.

Cattle genetic improvement schemes in Egypt have involved crossbreeding with exotic high producing cattle breeds such as Holstein, Brown Swiss, Friesian and Simmental (MoDAD 2004), in addition to Abondance and Tarentaise as dual purpose French breeds. Tarentaise cattle, descends from the French Savoie cattle, was chosen for its adaptability to tough conditions. It makes good use of rough forage, withstands temperature variation, adapt well to arid zone, perceived as hardy, proving robust and resistant, and recognized as easy-calving breed. This remarkable all-purpose blend of hardiness, milk and beef production has carried the breed's reputation abroad in North Africa, especially Tunisia and Egypt. Utilization of the Tarentaise breed in Egypt started in 1996 within the program called "Franco-Egyptian Development Plan for AI and Selection" during five years from 1996 to 2001, which was successful and therefore extendable for an extra five years; up till 2006. The program aimed to the production of 20,000 crossbred Baladi-Tarentaise (Bal-Tar) females for the improvement of cattle production in Egypt. The appearance of the Baladi-Tarentaise crosses resembles the Egyptian cows (Figure 1), reflecting the risk of uncontrolled gene flow to the native from the crossbred, and therefore the decay of pure native cattle genetic resources. Under such circumstances, studying the genetic variation and allele distribution within and among native and Bal-Tar cattle populations is crucial and time-effective. The findings of such study will contribute to the conservation and utilization of the native cattle genetic resources.

Microsatellite analyses have provided useful genetic information for African, European, mid-South American and Asian cattle breeds as reported by Dadi et al. (2008), Padilla et al. (2009), Acosta et al. (2013) and Pham et al. (2013), respectively. Microsatellites have been effectively exploited to evaluate genetic diversity among cattle populations (Sun et al. 2007).

The aims of this study were using DNA Microsatellite markers for studying the genetic diversity and structure of two major native cattle populations as Menufi and Saidi, and its crosses with Tarentaise breed, to identify genetic differentiation among the studied three Mediterranean cattle populations for the purpose of identification, utilization and conservation of indigenous native cattle genetic resources.

Figure 1. Visual appearance of Tarentaise cattle and their crosses with Egyptian Baladi cattle.

MATERIALS AND METHODS

Animals and sample collection

Blood samples representing unrelated animals were collected and utilized for obtaining genomic DNA from the two Egyptian native cattle populations and Bal-Tar crossbred (Figure 1). A total 97 random blood samples: 41 samples of Saidi cattle that were collected from Quena to Beni-sweif governorates at Upper Egypt, 21 samples of Menufi cattle that were collected from Zewair and Shanshour districts at Minufia governorate, and from El-Serw Research Station, Animal Production Research Institute. The 35 Bal-Tar crosses samples were collected from crosses with Saidi cattle from Quena to Beni-sweif governorates at Upper Egypt and crosses with Menufi cattle from El-Serw Research Station.

Egyptian cattle (Baladi) are medium-sized, long-bodied, lean of musculature and lightly boned. Medium length head, Face profile is straight or very slightly convex. Orbital arches are slightly accentuated, giving a small degree of concavity to the forehead. Poll is flat and Horns are short and grow from Poll laterally, curving forward so that their inclination is approximately at right angles to the line of the profile. Ears are moderate size. Neck is medium length and tends, in the female, to be lean. Dewlap and umbilical fold are small. Crest is accentuated in bull but it is only in the Saidi sub-type that a small cervico-thoracic hump is apparent in female. Rump is of very moderate slope and the accentuated tail setting is often higher than the withers. Body is long with only moderate depth and the ribs tend to be flat. Top-line dips in its central part between withers and prominent hook bones and bottom line rises from front to rear. Tail is moderate length. Thighs are flat and the limbs are long, lean and lightly boned. Coat coloration varies from fawn to red (Joshi et al. 1957).

Microsatellites genotyping

DNA was extracted from blood samples using Qiagen DNeasy® Blood and Tissue Kits according to manufacturers' instructions. DNA concentration was evaluated spectrophotometrically with Eppendorf® Biophotometer. Eight microsatellites including ETH10, ETH225, BM1818, BM1824, BM2113, SPS115, TGLA53 and TGLA126 as shown in Table (1) were genotyped in the 97 samples. Markers were selected for their reported polymorphism and allele size range from those recommended in Measurement of Domestic Animal Diversity (MoDAD 2004).

The eight microsatellites were amplified in two PCR multiplex reactions, each containing 100-150 ng templates DNA, 1X Platinum® Multiplex PCR Master Mix (Lifetechnologies®) and 10 pM of each primer (4 primer pairs in each multiplex) in 25 ul total reaction volume. The thermal profile followed by the ISAG/FAO Panel and was run on a C1000 Thermal

Table 1. Microsatellite markers genotyped and its observed allele size in studied animals

SSR (Chromosomal Location)	Observed allele size range, bp	Primer Sequence	Reference
BM1818 (D23S21)	258-280	F: AGCTGGGAATATAACCAAAGG R: AGTGCTTTCAAGGTCCATGC	Bishop & Kappes (1994)
BM1824 (D1S34)	179-193	F: GAGCAAGGTGTTTTTCCAATC R: CATTCTCCAACTGCTTCCTTG	
BM2113 (D2S26)	120-144	F: GCTGCCTTCTACCAAATACCC R: CTTAGACAACAGGGGTTTGG	
ETH10 (D5S3)	205-223	F: GTTCAGGACTGGCCCTGCTAACA R: CCTCCAGCCCACTTTCTCTTCTC	Toldo & Fries (1993)
ETH225 (D9S1)	139-157	F: GATCACCTTGCCACTATTTCCT R: ACATGACAGCCAGCTGCTACT	Steffen & Eggen (1993)
SPS115 (D15)	244-256	F: AAAGTGACACAACAGCTTCTCCAG R: AACCGAGTGTCCTAGTTTGGCTGTG	Moore & Byrne (1993)
TGLA53 (D16S3)	151-181	F: GCTTTCAGAAATAGTTTGCATTCA R: ATCTTCACATGATATTACAGCAGA	Georges & Massey (1992)
TGLA126 (D20S1)	117-129	F: CTAATTTAGAATGAGAGAGGCTTCT R: TTGGTCTCTATTCTCTGAATATTCC	

Cycler (Biorad ®). Genotyping and determination of single strand allele size for PCR amplicons were performed using the ABI3500 Genetic Analyzer (Lifetechnologies®) using the GeneScan® 600 LIZ® internal lane size standard. Allele size calling were carried out with GeneMapper (Applied Biosystems®).

Computations and statistical analysis

Frequencies and number of alleles for each locus, observed and expected heterozygosity, Wright's statistics F_{is} and F_{st} were estimated via FSTAT (Goudet 2001). GENEPOP (Raymond & Rousset 1995) was used to estimate Hardy–Weinberg equilibrium over loci within each population. Private alleles for any population was considered as those alleles unique for a population and detected in at least 25% of the sample of the population. The polymorphic information content (PIC) values were calculated according to Botstein et al. (1980).

Nei's (Nei 1987) standard genetic distances among populations were computed by POPGENE (Yeh et al. 1999). A pairwise matrix of the genetic distances was then used to obtain a Neighbor-joining (NJ) tree (Saitou & Nei 1987), which was visualized using the software FigTree (Rambaut 2012).

Bootstraps of 1000 replicates were performed in order to test the robustness of tree topology using the DISPAN (Ota 1993). The population structure was evaluated based on a Bayesian clustering analysis by employing structure (Pritchard et al. 2000), using multi-locus genotypes to infer for all the individuals and populations for the fractions in their genetic ancestry that belong to a given number (k) of clusters. A Monte Carlo Markov chain was run for k = 2 to 3, with a burn-in period of 20,000 and a run length of 20,000 iterations. A default setting assuming an admixture model with correlated allele frequencies was used in all runs. The Delta K approved that the optimum K is 3.

RESULTS AND DISCUSSION

Allelic distribution and private allele

Table 2 shows that microsatellites used in the present study were polymorphic for the three populations studied which revealed the existence of considerable genetic variability among the animals sampled. The highest number of alleles was 16 at TGLA53 and the lowest was 6 at SPS115. Bal-Tar cross had the highest total and mean number of alleles over all loci as 68 and 8.5±3.8 alleles, respectively; which should be due to recombination of first generation crossbred. Saidi and Menufi populations also showed less but considerable number of alleles as 64 and 62 alleles, respectively. Studied populations showed presented genetic polymorphism proved by the considerable detected high total and mean number of alleles. Most loci proved to be highly polymorphic in native and its crosses. These results were in agreement with a mean 8.4 alleles per locus obtained by MacHugh et al. (1997) in Taurine and Zebu cattle populations, while it was higher than the mean of 6.5 alleles per locus reported by Beja-Pereira et al. (2003) in Iberian and French cattle breeds.

Obtained results are higher than those reported by Molina-Flores et al. (2011) for Saidi cattle being 6.25 alleles using 28 microsatellites and less than what reported by Hassanane et al. (2006), being 9 alleles in five local Egyptian populations in Delta, North and Mid-Egypt. Also, Cymbron et al. (2005) worked on different cattle populations utilizing 19 microsatellite loci and found much lower means number of alleles were 5.57±0.2, 3.81±0.15 and 4.29±0.16 alleles for 32 animals of Egyptian native population, 38 animals of Salers and 27 animals of Vosges as French breeds, respectively.

In this study one private allele (PA) was found in Menufi population with frequency =0.25 and size=*205* bp for ETH10 marker and two private alleles were found in Bal-Tar cross with frequency = 0.37, size=191 bp for BM1824 marker and frequency = 0.27, size=119 bp for TGLA128 marker as presented in Table (2).

Luo et al. (2006) used ten SSR to detect the genotypes of ten Chinese cattle breeds and 3 introduced breeds, the results showed that there were 117 alleles from the 10 SSR loci in 13 populations, which alleles of HEL9 carry the most number (18), ILSTS01 was the least (7). Number of alleles in the remaining seats was between 8 and 14, Dominant alleles of 13 Chinese and foreign breeds were mainly composed of ILSTS011, INRA035 and ILSTS005.

Suh et al. (2014) reported that a total of 276 alleles were detected at 30 microsatellite loci across four Korean native cattle breeds (Hanwoo, Chikso, Heugu, and Jeju black). The total number of alleles per locus ranged from 4 (ILSTS005) to 17 (TGLA122), with a mean of 9.20±0.58 alleles.

Polymorphic information content (PIC)

The PIC is a parameter indicative of the informative degree of a marker. The PIC values range from zero to one. Loci with many alleles and higher PIC values up till one are the most desirable markers and PIC of >0.5 indicates a highly informative locus for chromosomal mapping and genetic diversity (Botstein et al. 1980). Microsatellites display a high degree of polymorphism, with a mean PIC of 0.6 (Vaiman et al. 1994). Most of the loci were highly informative (PIC>0.5), with the exception of ILSTS005 (0.375) and HEL13 (0.413). Similarly, ILSTS005 and HEL13 have been reported to

be the relatively low informative markers (Padilla et al. 2009).

The PIC values for all the eight markers are shown in Table (2). Average of PIC values for the eight microsatellites was 0.696, ranged from 0.238 for SPS115 to 0.898 for TGLA53 in native cattle; and averaged 0.725, ranged from 0.476 for SPS115 to 0.873 for TGLA53 in Bal-Tar cross. The polymorphism information content values of the seven from eight observed loci are tending to be high (PIC >0.5) in the studied populations. The highest PIC values were 0.898, 0.866 and 0.873 in TGLA53 marker in Saidi, Menufi and Bal-Tar cross, respectively; while the lowest PIC values of SPS115 marker were 0.367, 0.238 and 0.476 respectively for the same populations.

Average PIC values in this study, was in close agreement with those reported by Hassanane et al. (2006) working on five local Egyptian cattle populations; where PIC varied from 0.716 for INRA 05 marker of cattle population that locate at Delta district to 0.883 for HEL09 marker of cattle population that locate at Domiatt district. PIC was averaged 0.720±0.02 across cattle breed groups includes Angus, Brangus, and their crosses with Brahman using ETH10 (DeAtley et al. 2011).

Qiu (2007) analyze the genetic diversity of the Xiangxi cattle used 6 SSR and found 65 alleles in total. PIC average of 6 SSR loci in Xiangxi cattle was more than 0.5, which indicated that the genetic diversity of Xiangxi cattle was rich.

Agung et al. (2016) reported that the PIC value of the 12 observed loci is high (PIC>0.5) and the highest PIC value in the Simmental cattle population was 0.893 for locus TGLA53. Kesvulu et al. (2009) reported that the overall mean estimate of PIC was 0.628 and it ranged from 0.308 for ETH225 to 0.809 for TGLA122

Intra-breed genetic variation

Table 3 shows means of observed heterozygosity (H_{obs}) and expected heterozygosity (H_{exp}) as gene diversity values, the chi-square test for Hardy-Weinberg equilibrium (HWE) and Wright's F_{is} in studied populations. Within breed genetic variability is relatively high, as evidenced by the high mean expected heterozygosity (H_{exp}= 0.75).

Table 2. Number of alleles, PIC, PA (Size in bp, Freq.), and n_a of the studied SSR

SSR Marker	Term	Cattle populations			Total Number of alleles
		Saidi	Menufi	Bal-Tar	
BM1818	No of alleles	8	8	10	10
	PIC	0.693	0.712	0.795	
BM1824	No of alleles	5	5	5	7
	PIC	0.689	0.608	0.667	
	PA			(191:0.367)	
BM2113	No of alleles	9	12	12	13
	PIC	0.760	0.846	0.841	
ETH10	No of alleles	9	10	8	10
	PIC	0.839	0.828	0.651	
	PA		(205:0.250)		
ETH225	No of alleles	8	8	8	9
	PIC	0.773	0.752	0.741	
SPS115	No of alleles	6	3	3	6
	PIC	0.367	0.238	0.476	
TGLA53	No of alleles	14	12	15	16
	PIC	0.898	0.866	0.873	
TGLA126	No of alleles	5	4	7	7
	PIC	0.670	0.592	0.752	
	PA			(119:0.271)	
Total	n_a±SD	8±2.9	7.75±3.5	8.5±3.8	9.75±3.4
	No of alleles	64	62	68	78±2.2

PIC= Polymorphic information content, **PA** = Private alleles, n_a = Mean of observed number of alleles.

Table 3. Estimates of intra-breed genetic variation of observed and expected heterozygosity and Hardy Weinberg Equilibrium for the studied populations

Populations	№	Hobs± SE	Hexp± SE	HWE test	Fis
Saidi	41	0.71 ±0.025	0.75 ±0.055	0.0005	0.048
Menufi	21	0.62 ±0.038	0.73 ±0.073	0.0000	0.148
Bal-Tar cross	35	0.86 ±0.021	0.77 ±0.040	1.0000	-0.118
All	97	0.73 ±0.028	0.75 ±0.056	---	0.026

H_{obs} = Mean of observed heterozygosity, **H_{exp}=** Mean of expected heterozygosity, **HWE**= Hardy-Weinberg equilibrium, **F_{is}**= Wright's F_{is}

The present means of H_{exp} and H_{obs} for Bal-Tar cross were 0.77 and 0.86, respectively; these results were the highest values among the three studied populations, which might be due to recombination of F1 crossbreeding. Also the crossbred population is the only one showing negative value of heterozygosity deficiency estimate as a result of outbreeding strategy in mating. These results were higher than those reported for pure Tarentaise by Maudet et al. (2002), who found that means of H_{exp} and H_{obs} were 0.699 and 0.685, respectively. On the contrary, mean observed heterozygosity was lower than the expected and F_{is} were positive in the two native Egyptian populations indicating heterozygosity deficiency it may be due to uncontrolled inbreeding as a mating strategy at these areas for a long time.

Deviation from HWE was significant in the two Egyptian populations and non-significant in the Bal-Tar cross. Mean values of heterozygosity estimates in native cattle were higher in Saidi than Menufi (Table 3), and the Menufi cattle showed the least number of observed heterozygosity (0.62). These results indicated that Menufi population has the least genetic variability and the highest inbreeding coefficients among the populations studied.

Numbers of Menufi cattle population decreases and relying on village-bull for insemination increases the chance of inbreeding. For the Egyptian populations, Hassanane et al. (2006) reported a general higher H_{exp} that ranged from 0.813 to 0.858 in the five local cattle populations studied. This study was ten years ago, during which period, the native cattle populations in delta significantly changed. The high genetic diversity observed in a population could be explained by overlapping generations, mixing of populations from different geographical locations, natural selection favoring heterozygosity or subdivision accompanied by genetic drift. The effect of these factors is more pronounced when the effective population size is very large.

Cymbron et al. (2005) reported that the estimates of expected heterogozity H_{exp} were 0.77, 0.55 and 0.68 for 32 animals of Egyptian native population, 38 animals of Salers and 27 animals of Vosges as French breeds, respectively. Kesvulu et al. (2009) reported that the overall mean observed and expected heterozygosities were 0.684 and 0.666, respectively and ranged from 0.304 to 1.0 and 0.334 to 0.829, respectively, indicating higher polymorphism of the microsatellite loci in the population of Punganur cattle. Suh et al. (2014) reported that the mean of H_{Exp} across loci was 0.733±0.018, with estimates per locus ranging from 0.473 (ILSTS005) to 0.893 (TGLA53). For H_{Obs}, the mean for all loci was 0.667±0.028, and the range was between 0.174 (INRA035) and 0.855 (CSRM60).

Chaudhari et al. (2009) reported that the Means of observed and expected heterozygosity were found to be 0.47±0.24 and 0.62±0.21 in Kenkatha, and 0.53±0.17 and 0.68±0.14 in Gaolao cattle in India, respectively. Heterozygosity deficit within a population was measured by Wright's F_{is}. Positive F_{is} values in Menufi and Saidi indicate that individuals in these populations are more related than expect under a model of random mating and were higher (F_{is} =0.148) in Menufi than its value for Saidi (F_{is} =0.048) as reported in Table (3), indicated higher inbreeding coefficients in Menufi than Saidi breeds. Negative F_{is} values in Bal-Tar cross (F_{is} =-0.118) indicate that individuals in this population are less related than expect under a model of random mating.

Genetic differentiation among breeds

Table 4 shows that pairwise genetic differentiations quantified by F_{st} estimates ranged from 0.006 between Saidi and Menufi to 0.085 between the Bal-Tar cross and Menufi. Similarly Nei's (Nei 1987) standard genetic distance ranged between 0.074 between Saidi and Menufi, and 0.393 between Bal-Tar cross and Menufi. Low estimate of genetic differentiation (F_{st}) between the two Egyptian populations reflects high genetic similarity between these breeds. Hassanane et al. (2006) reported Fst estimates, between five indigenous cattle populations, that its absolute value ranged between 0.001 to 0.046, while Molina-Flores et al. (2011) studying Saidi cattle, reported an average F_{st} estimate of 0.018 among different Saidi cattle population. All identified SSR alleles in this study

Table 4. Estimated genetic differentiation [(above diagonal)] and genetic distance [(below diagonal)] among studied populations

Populations		Saidi	Menufi	Bal-Tar cross
Egyptian native cattle	Saidi	-	0.006	0.069
	Menufi	0.074	-	0.085
French-Egyptian cross	Bal-Tar	0.305	0.393	-

[#] Pairwise F_{st} as a measure of genetic differentiation. [##] Nei's (Nei 1987) standard genetic distance

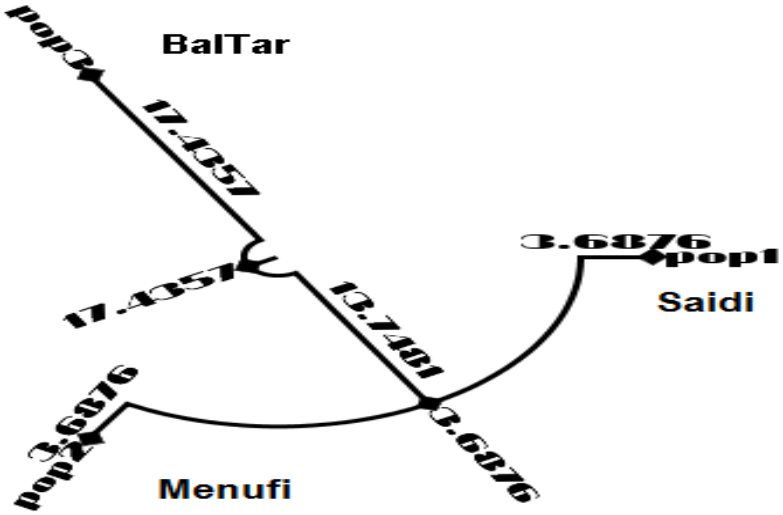

Figure 2. Dendrogram showing the genetic relationship among the three populations of cattle using Ds genetic distance.

were able to classify the studied populations into groups. There are subpopulations that are closely related and form their own group. When the population relationships are visualized with the Dendrogram (Figure 2), Egyptian Menufi and Saidi are together at the same cluster with a low bootstrap value (20,000). Both Egyptian Menufi and Saidi cattle are Nile valley and delta populations. There is only weak differentiation among the Egyptian populations, while the phylogenetic tree shows a clearer differentiation among the native and Bal-Tar cross (Figure 2). Thus, three genetic clusters were identified, that is, two monophyletic clusters coinciding; one Bal-Tar cross and a cluster of the two Egyptian populations. Geographical distribution of (distances among) the three populations is in consistent with the phylogenetic dendrogram (Figure 2). Our results indicated that genetic components of the two Egyptian cattle populations have high level of similarity, which can be due to little genetic divergence among them after their immigration into Egypt out of the center of domestication in the Near East.

Information about the genetic distance in this study confirms Hassanane et al. (2006) who reported that in general gave evidence that all Baladi cattle raised in Egypt have genetic similarities, and could be considered as a one breed. This does not agree with (FAO 1993), which reported that Egyptian cattle have many breeds. The variations in productivity of some populations especially between Domiatt and Saidi, may be due to environmental or management factors.

Genetic structure

The population structure and the level of admixture in the cattle populations were analyzed by using structure software (Pritchard et al. 2000) which is a model-based clustering analysis, for a k ranged between 2 to 5. According to the structure results and the Delta K estimates, the most probable number for K was 3 ancestral populations (Figure 3 and 4). The structure analyses generate similar interpretation with the dendrogram (Figure 2).

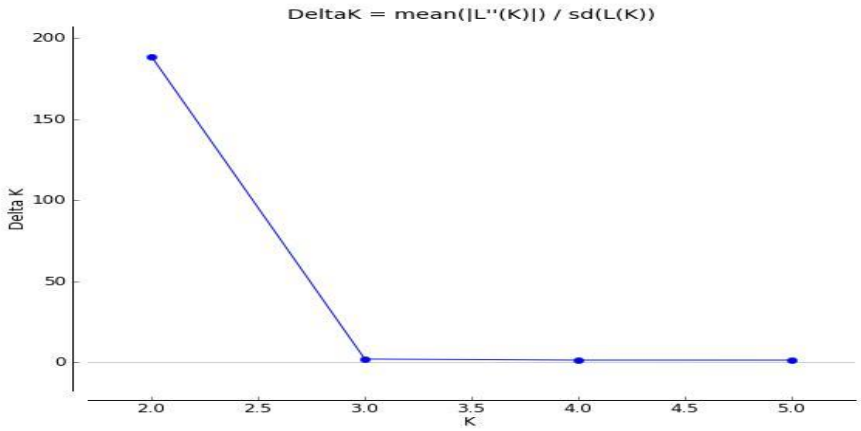

Figure 3. The Delta K estimate.

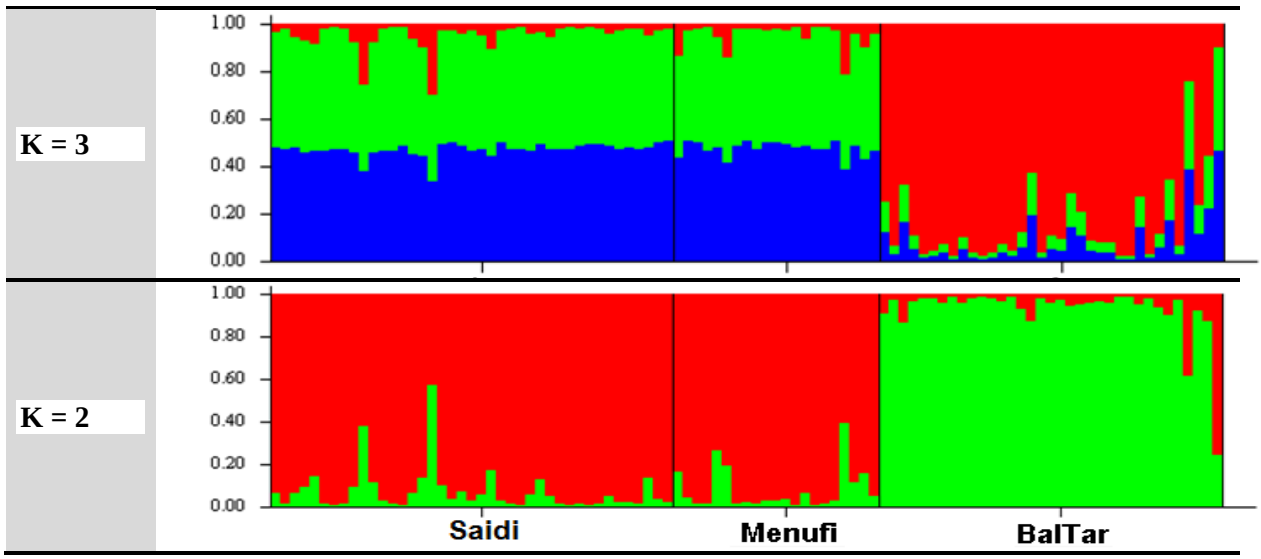

Figure 4. Genetic structure of the studied cattle populations.

Figure 4 shows that structure analysis indicated three ancestral genetic backgrounds. The two native breeds share two main backgrounds in almost equal percentages, while the Bal-Tar cross has the third one. The three populations showed low percentage of admixture with other backgrounds (Figure 4). Such admixture might reflect the inheritance from the common ancestor's from the center of domestication and their movement to the Mediterranean region (South European and North Africa).

Figure 4 shows the genome distribution for each individual in both inferred clusters (K). Each animal is represented by a single vertical bar. The length of the bar color in the vertical axis defines the membership proportion (Q). Where k is the number of clusters assumed and the length of the colored segment represents the individual's estimated proportion of membership to a particular cluster. Luo et al. (2006) the genetic structure between Yanbian cattle, Yajiang cattle and Changbai local cattle is very similar.

CONCLUSION

The SSR markers utilized as a part of this work were appropriate in surveying genetic diversity and structure in the Egyptian and crossbred cattle populations analyzed, uncovering elevated amounts of genetic variability. Egyptian cattle populations studied; Egyptian Menufi and Saidi; deviate from HWE and suffer from heterozygosity deficiency, indicating levels of inbreeding, which might be due to decreasing number of reproducing animals and village-cull breeding system. The study indicates that the two

Egyptian populations and their crosses with the French Tarentaise breed can be genetically differentiated, in line with their geographical distribution and the crossbreeding.

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Plant Extract Activities as Antioxidant and Antibiofilm against Chicken Gut Bacteria

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ABSTRAK

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Terjadinya resistensi mikroba terhadap antibiotik akibat penggunaan *antibiotic growth promoter* (AGP) dengan dosis subterapeutik pada unggas dapat dicegah dengan senyawa antibiofilm. Senyawa sekunder tanaman memiliki aktivitas seperti antioksidan, antimikroba, maupun antibiofilm. Penelitian ini bertujuan mendapatkan tanaman yang memiliki aktivitas antibiofilm tertinggi dan juga aktivitas antioksidan dan dengan cara menganalisis aktivitas senyawa sekunder beberapa tanaman. Sampel tanaman yang diuji adalah daun cengkeh, tanaman meniran, kulit manggis, cangkang jambu mete, daun jambu, dan daun salam. Tanaman tersebut diekstraksi dengan metanol dan n-heksana menggunakan metode sonikasi. Aktivitas antioksidan ekstrak metanol tanaman diukur dengan mencari nilai IC_{50} pada uji α,α-diphenyl-β-picrylhydrazyl (DPPH). Aktivitas inhibisi pembentukan biofilm diuji terhadap *Escherichia coli*, *Salmonella enteritidis*, dan *Staphylococcus aureus* ATCC® 29213™ menggunakan ekstrak metanol dan n-heksana. Seluruh sampel memiliki aktivitas antioksidan. Sampel daun cengkeh dan tanaman meniran memiliki aktivitas antioksidan tertinggi. Sementara, ekstrak metanol kulit manggis memiliki aktivitas antibiofilm tertinggi terhadap seluruh bakteri uji. Jenis bakteri uji juga mempengaruhi aktivitas antibiofilm. *E. coli* dan *S. enteritidis* lebih resisten terhadap antibiofilm dibandingkan *S. aureus.* Ekstrak kulit manggis memiliki aktivitas antibiofilm dan antioksidan yang tinggi sehingga berpotensi untuk digunakan sebagai pakan imbuhan untuk mengendalikan bakteri patogen dalam saluran pencernaan unggas.

Kata Kunci: Ayam, Tanaman, Senyawa Sekunder, Antioksidan, Antibiofilm

ABSTRACT

Gracia E, Magdalena S, Wina E, Sinurat AP, Purwadaria T. 2018. Plant extract activities as antioxidant and antibiofilm against chicken gut bacteria. JITV 23(1): 11-17. DOI: http://dx.doi.org/10.14334/jitv.v23i1.1800

The occurrence of microbial resistance against antibiotic due to the subtherapeutic dosage of antibiotic growth promoter (AGP) in poultry can be prevented by the antibiofilm substance. Plant secondary compounds have some activities like antioxidant, antimicrobial, and antibiofilm. This research was conducted to obtain the plant with the highest activity of antibiofilm and also antioxidant by analyzing several plant secondary compounds as antioxidant and antibiofilm against chicken's gut bacteria. The tested plants were clove leaves, leaffruit plants, mangosteen peel, cashew nut shell, guava leaves, and bay leaves. These plants were extracted with methanol or n-hexane using sonication method. The antioxidant activity as the IC_{50} value of the plant methanol extracts were determined using α,α-diphenyl-β-picrylhydrazyl (DPPH) assay. The biofilm inhibition activity was tested against *Escherichia coli*, *Salmonella enteritidis*, and *Staphylococcus aureus* ATCC® 29213™ using methanol and n-hexane extracts. All of the samples had antioxidant activity. The clove leaves and leaffruit plants had the highest antioxidant activity, while mangosteen peel extract in methanol had the highest antibiofilm activity against all tested bacteria. The species of bacteria also affected the antibiofilm activity. *E. coli* and *S. enteritidis* were more resistant to antibiofilm then *S. aureus*. Mangosteen peel extract which showed high antioxidant and antibiofilm activity is potential to be used as a feed additive to control the pathogenic bacteria.

Key Words: Chicken, Plant, Secondary Compounds, Antioxidant, Antibiofilm

INTRODUCTION

In poultry industries, pathogenic bacteria like *Escherichia coli*, *Salmonella enteritidis* (both Gram negative), and *Staphylococcus aureus* (Gram positive) often cause severe illness. These industries have been using subtherapeutic dosage of antibiotic growth promoter (AGP) in their poultry feed to kill the bacteria and increase poultry feed efficiency for more than 5 decades (Lin et al. 2013). The use of AGP as feed additive will decrease good gut bacteria bile salt hydrolase (BSH) enzyme activity, such as *Lactobacillus* spp. which takes part in poultry lipid metabolism (Lin 2014).

Practically, the AGP supplementation doesn't kill the pathogenic bacteria effectively due to their ability to form biofilm which is a complex community of microbial cells that are associated with a surface and enclosed in a self-produced biopolymer matrix. The biofilm cannot be easily penetrated by the AGP. Such structured community is a response of bacteria to a signal system named quorum sensing (QS) constituted by inter-cell Therefore, the AGP will only kill the planktonic bacteria (Høiby et al. 2010). The subtherapeutic AGP also leads the bacteria to produce antibiotic resistance gene (Lin et al. 2013). Besides that, the mutation can be occurred due to the oxidative stress of the bacteria colony in the biofilm. When the bacteria had the oxidative stress, the bacteria will be mutated to survive. One solution to control the oxidative stress and decrease the mutation is antioxidant addition (Høiby et al. 2010).

The structure of biofilm matrix caused gradient of oxygen and nutrient occur in biofilm matrix and it is related to the different kind of growth rate of the biofilm active growing and biofilm persistent microbes (del Pozo & Patel 2007). Biofilm active growing microbes grow faster than the biofilm persistent microbes because they are outside of the colony, where the oxygen and nutrition supplies are better. Less oxygen and nutrition supplies in deeper biofilm matrix caused the biofilm persistent microbes have lower metabolism and slower growth rate. To kill the bacteria inside the biofilm or the persistent bacteria need to be noticed. The biofilm structure that cannot be easily penetrated by the antibiotic also protected the bacteria inside from being killed also caused the bacteria inside hard to kill. Another problem is the antibiotic resistance gene that produced by the bacteria easily transferred among the bacteria due to the bacterial communication (QS) inside the biofilm matrix. The communication also can change the non-pathogenic bacteria to pathogenic bacteria. Therefore, antibiofilm substance addition is important to control the pathogenic bacteria (Bjarnsholt et al. 2013).

Naturally, some of higher plants have the ability to produce secondary compounds which have some activities like antioxidant (Bjarnsholt et al. 2013), antimicrobial (Budzyńska et al. 2011), or antibiofilm (Geethashri et al. 2014). Secondary compounds in clove leaves had antimicrobial and quorum quenching (anti QS) activities (Aparna et al. 2014); in leaffruit plants had anticancer, anti-inflammation, and antibacterial activities (Sarin et al. 2014); in mangosteen peel had antioxidant, antimicrobial, antifungal, antiviral, and anti-inflammation activities (Palakawong et al. 2010); in cashew nut had antifungal and antibacterial activities (Harlita et al. 2016); in guava leaves had antimicrobial, quorum quenching, and anti-inflammation activities (Lazar et al. 2013; Biswas et al. 2013); and in bay leaves had antibacterial, antifungal, and antioxidant activities (Kusuma et al. 2011). Secondary compounds might be used simultaneously with AGP as feed supplements. The compounds will inhibit the biofilm formation, while the AGP will kill the pathogen bacteria. This condition will make the subtherapeutic AGP addition become effective and not result in any resistance. Therefore, the benefit of AGP application might be maintained and permitted. This research was conducted to discover the activities of plant secondary compounds as antioxidant and antibiofilm against chicken gut bacteria.

MATERIALS AND METHODS

Plant materials

This research examined the secondary metabolites activity of clove leaves (*Syzygium aromaticum*), leaffruit plants (*Phyllanthus urinaria*), mangosteen peel (*Garcinia mangostana*), cashew nut shell (*Anacardium occidentale*), guava leaves (*Psidium guajava*), and bay leaves (*Syzygium polyanthum*). These samples were collected, dried and powdered by Indonesian Research Institute for Animal Production at Ciawi, West Java.

Source of test microorganisms

Pathogenic cultures used in this research are *E. coli*, *S. enteritidis*, and *S. aureus*. Pure cultures of *E. coli* and *S. enteritidis* were isolated from the poultry gut and obtained from Sri Laboratory, Bogor, while the pure culture of *S. aureus* ATCC® 29213™ was obtained from Atma Jaya Catholic University of Indonesia, Jakarta.

Preparation of plant extracts

All of the plant samples were extracted with methanol and n-hexane. The methanol extract was used for antioxidant and antibiofilm assays, while the

n-hexane extract only for antibiofilm assay. The stock extract solution for each sample was carried out by soaking 0.5 gram powdered sample in 10 mL methanol or hexane and macerated in sonication water bath for 30 minutes. Then, the extract was filtered with filter paper (Annegowda et al. 2010). The methanol extract was then diluted to appropriate solution for antibiofilm assays.

For antibiofilm assay samples of sonicated extracts in methanol or n-hexane were evaporated using rotary evaporator at 40 ˚C (Annegowda et al. 2010) until dry and diluted with 2 mL of 10% dimethyl sulfoxide (DMSO) into extract concentration of 50 mg/mL. After that, all filtrates were filtered using 0.2 µm non-polar filters (Selim et al. 2014).

Preparation of bacterial cultures

Stock cultures were stored in the mixture of tryptic soy broth (TSB) and 87% glycerol (4:1) at -80 ˚C. While working cultures were maintained in nutrient agar (NA) and kept at 4 ˚C. In each experiment, the cultures were refreshed in NA overnight at 37 ˚C. After that, the bacteria were prepared in broth medium. *E. coli* and *S. enteritidis* were inoculated in TSB, while *S. aureus* in TSB with 2% glucose and 2% sucrose overnight at 37 ˚C (Djordjevic et al. 2002).

Antioxidant activity assay with α,α-diphenyl-β-picrylhydrazyl (DPPH)

DPPH solution was diluted in methanol (0.2 mM). A series of different concentrations of samples was prepared from stock extracts to obtain 50% inhibition depending on the kind of plant extracts. To determine the antioxidant activity, 1 mL of sample was reacted with 2 mL DPPH solution. As control, 2 mL DPPH solution was reacted with 1 mL methanol. Ascorbic acid (5-20 ug/ml) was also used as the reference. All mixtures were incubated at room temperature in the dark condition for 30 minutes. The absorbance of each sample was measured at 517 nm wavelength. The blank used in this measurement was methanol. As control, 1 ml of methanol was reacted with 2 ml of DPPH solution. The DPPH inhibition activity of each sample was calculated towards control absorbance, while IC_{50} value was determined in the curve of DPPH inhibition percentage towards plant extract concentration (Shekhar & Anju 2014).

$$\% \text{ DPPH Inhibition} = \frac{X - Y}{X} \times 100\%$$

X = Control absorbance
Y = Sample Absorbance

Biofilm inhibition activity assay

The absorbance of each bacterial culture prepared in the broth medium was measured at 600 nm wavelength to reach 0.132 (0.5 McFarland). The bacterial cultures were inoculated as much as 180 µL together with 20 µL plant extracts in 96-wells polystyrene microplates (Iwaki) overnight at 37˚C. As positive control, 180 µL cultures were inoculated together with 20 µL sterile 10% DMSO. However, the negative control only used 200 µL broth medium without bacterial culture. For biofilm inhibition activity against *S. aureus*, the methanol extract's concentrations were diluted 10 times.

After the incubation time, medium and planktonic cells were removed and the plate was washed with distilled water twice with flow through method. Then, the plate was air dried for the staining for 15 minutes. Each well was stained with 200 µL 0.4% crystal violet in ethanol for 30 minutes. Crystal violet was then removed and the plate was washed with distilled water three times with flow through method. The plate was air dried again and added with 200 µL ethanol for 30 minutes. The blue solution appeared as the biofilm expression and the solution was transferred to the new microplate and the absorbance of each well was measured with BIO-RAD Model 680 Microplate Reader at 595 nm wavelength. Biofilm inhibition activity was measured with this equation below (Djordjevic et al. 2002).

$$\text{Biofilm inhibition} = \frac{A - B}{C} \times 100\%$$

A = Positive Control
B = Sampel absorbance
C = Positive Control absorbance

RESULTS AND DISCUSSION

Antioxidant activity

The samples concentration for the most suitable IC_{50} value was varied depending on the sample source (Table 1). The highest IC_{50} value means the lowest antioxidant activity. Based on the IC_{50} value data, it seems that there were two groups of antioxidant activity. The first group with the high antioxidant activity (275-360 µg/mL) were cashew nut shell, mangosteen peel, leaffruit plants, and clove leaves, while the second with low antioxidant activity (> 600 µg/mL) were bay leaves and guava leaves. However, the samples with high antioxidant activity had significantly lower antioxidant activity compared to the ascorbic acid which had 14 µg/mL IC_{50} value. It was

Table 1. Antioxidant sample concentration and IC_{50} value

Samples	Concentration range (µg/mL)	IC50 Value (µg/mL)
Bay leaves	500-800	760
Guava leaves	400-800	626
Cashew nut shell	100-500	360
Mangosteen peel	200-350	338
Leaffruit plants	100-500	292
Clove leaves	100-400	275
Ascorbic acid*	5-20	14

* Ascorbic acid was used as a reference.
**These antioxidant activities were also correlated with their anti bacteria and antifungi activities in Sinurat et al. (2017).

possible since ascorbic acid is a pure compound having very high antioxidant activity, while the plant extracts contained mixture of secondary compounds. Purification of the compound in plant extract might increase the activity.

The low reactivity of DPPH to hydrophobic antioxidant and pH dependent were the limitations in this assay. Different solvent would cause different results because the reactions of the DDPH were different in every solvent (Sultana et al. 2009). The use of methanol as solvent in this experiment also affected the results. Reactions of phenolic compounds were faster in methanol compared to ethanol or acetone. Since the major secondary compounds of the samples were phenolic compounds, the results showed high antioxidant activity (Xie & Schaich 2014).

Biofilm inhibition activity

All methanol extract samples showed biofilm inhibition to all tested bacteria, except cashew nut shell which is negatively against *E. coli* (Table 2). This condition might occur because the oily compound in the extract adhered to the surface and made the absorbance measurement invalid. Among all methanol extract samples, mangosteen peel had the highest biofilm inhibition activity against all tested bacteria.

Methanol extracts for *S. aureus* were diluted 10 times with sterile 10% DMSO because the antibiofilm activity against *S. aureus* already detected at low concentrations. The 10 times dilution of the filtrates for *S. aureus* showed that *E. coli* and *S. enteritidis* were more resistant compared to *S. aureus*. The data showed that all methanol extract samples had antibiofilm activities and mangosteen peel extract had the highest antibiofilm activity (Table 2). The ability of mangosteen peel as antibiofilm was related to the α-mangostin (xanthone) contained in it. As reported by Nguyen et al. (2015), its antibiofilm activity related to the inhibition of glycosyl-transferases which associated with the formation of the EPS matrix.

Among all methanol extract samples, cashew nut shell liquid had no antibiofilm activity against *E. coli*. The negative result occurred could not be easily described, since the extract containing anacardic acid known as antibacterial compound. The possible explanation is the acid or the oily compound might be used by *E. coli* to form biofilm matrix (Rodrigues 2014). However, it was still unclear whether the oily compound which interfere the absorbance measurement was biofilm matrix or not.

All the n-hexane extracts did not show antibiofilm activity as high as the methanol extract (Table 2). The n-hexane extract samples did not show any biofilm inhibition activity against *S. aureus*. The n-hexane extract samples only showed antibiofilm activity against *E. coli* (leaffruit plants) and *S. enteritidis* (cashew nut shell). This condition might occur due to the sterol and triterpene contained in leaffruit leaves, such as β-sitosterol, β-amyrin, methyl gallate, and trimethyl 1-3,4 dehydrochebulate (Sarin et al. 2014). The ability of cashew nut shell as antibiofilm agent might occur due to the secondary metabolites contained in it which could be extracted in n-hexane. Those secondary metabolites were monounsaturated anacardic acid, β-sitosterol, monounsaturated cardol, saturated cardol, di-unsaturated cardol, and triacontene (Taiwo 2015). The previous study on anaerobic gas production method showed that cashew nut shell extract in n-hexane had antimicrobial activity against poultry gut bacteria (Sinurat et al. 2018). Biofilm inhibition assay method used in the research had some limitations. One of them was the single culture used may not behave or react like the mixed population found in natural environment. Another limitation was microtiter plate itself had different surface compared to the natural environment. Regardless, this method is a widely used tool in the study of biofilm. In this research, the composition of the

Table 2. Biofilm inhibition percentage against tested bacteria

Sample	Inhibition Percentage (%)		
	E. coli	*S. enteritidis*	*S. aureus**
	Methanol extract		
Bay leaves	44.34 ± 3.89	60.22 ± 2.86	85.87 ± 2.15
Guava leaves	49.94 ± 2.38	74.32 ± 1.02	88.47 ± 0.89
Cashew nut shell	**	72.05 ± 1.64	94.71 ± 0.48
Mangosteen peel	62.86 ± 2.30	81.63 ± 1.66	95.28 ± 0.54
Leaffruit plants	47.68 ± 2.86	76.49 ± 3.90	88.48 ± 0.93
Clove leaves	53.24 ± 3.73	79.40 ± 1.72	73.30 ± 2.07
	n-Hexane extract		
Bay leaves	**	**	**
Guava leaves	**	**	**
Cashew nut shell	**	11.80 ± 1.12	**
Mangosteen peel	**	**	**
Leaffruit plants	28.65 ± 6.76	**	**
Clove leaves	**	**	**

* The concentration of methanol extracts for *E. coli* and *S. enteritidis* was 10 times higher than for *S. aureus*.
** Negative results.

media was an important thing because it affected the biofilm formation. The composition of the media was the leading role in forming the biofilm matrix. For *S. aureus* biofilm formation, as reported by (Skyberg et al. 2007), the addition of sugar caused higher biofilm formation (data not shown). However, the addition of sugar for *E. coli* and *S. enteritidis* made the bacteria not forming the biofilm matrix. To get comparable antibiofilm data for each bacterium, optimisation of the media and its microenvironment should be carried out (Skyberg et al. 2007).

All samples had antioxidant and antibiofilm activities. The antioxidant compounds should take the role in inhibition process in all pathogenic bacteria, however, in this experiment not all high antioxidant activities were followed with high antibiofilm activities. When we correlated both activities statistically, there was only one significantly correlation between antioxidant activities of all plant methanol extracts and antibiofilm activities towards *S enteridis* with R^2 = 67%; those from other bacteria and extracts did not significantly correlated. It was reported that the antibiofilm compound of plant secondary metabolites is preventing the communication among the bacteria to build the biofilm (Koh et al. 2013) and it would decrease the mutability of the bacterial cells inside the matrix, especially the ability to form the antibiotic resistance gene. The antibiofilm activity expressed differently in each bacterium might occur due to a specific correlation between each bacterium and the antibiofilm activity of each plant extract. The antioxidant activity of mangosteen peel was quite high to take the role in antibiofilm activity.

In the test of anti-microorganism growth (antibacteria) by *in vitro* fermentation method or by measuring the total gas production, all plant extracts using methanol and hexane inhibited the gas production or the growth of bacteria. However, using clearing zone test only methanol extracts of guava leaves, cashew nut shell, mangosteen peel, and clove leaves showed inhibition activity to *E. coli* but no inhibition to *S. enteritidis*, while the extracts of leaffruit plants and bayleaf did not inhibit the growth of both bacteria. In the hexane extracts, only *S. enteritidis* was inhibited by guava leaves, mangosteen peel, and leaffruit plants, the extracts of cashew nut shell, bay and clove leaves did not form any clearing zones (Sinurat et al. 2017). The antibacteria activity of the plant extracts might affect the antibiofilm activity, however, the gas formation test mimics the digestion system which contain enormous kind of bacteria. Therefore, the data cannot be compared to the antibiofilm activity towards specific pathogen bacteria like in our experiment. Results of clearing zone formations which used same pathogenic bacteria and same concentration of plant extracts may indicate the false conclusion of antibiofilm activity. However, these data were observed from different method. In the top of that a lot of extracts which showed antibiofilm activity did not show antibacterial activity except for methanol extracts of mangosteen

peel, guava and clove leaves for *E.coli*. All other positive antibiofilm activities were not influenced by antibacterial activity. Determinations of antibacterial together with antibiofilm activities were suggested by Er et al. (2014). They reported that some food or feed preservatives such as ciprofloxacin, sodium nitrite and potassium sorbate had more antibacterial than those antibiofilm activities which should be considered for the possibility the cause of resistency.

The antibiofilm activity of the plant extracts will be useful as feed supplement that preventing the quorum sensing of bacterial pathogenic bacteria to form the colonies inside the biofilm or only in the planktonic cell condition. This condition may result the effectivity of the AGP as bacterial killer. At the end no resistant cells will be formed. For future study, the optimum concentration as feed supplement needs to be calculated in relation to replace AGP so the supplementation becomes effective. It would be more interesting if the extracts could replace all the AGP supplementation to reduce the supplementation cost and also to prevent the occurrence of antibiotic resistance genes. For more effective supplementation, probably it needs to purify the extract to get the specific antioxidant and antibiofilm compounds with high activity. Furthermore, it is important to test the effectivity of the pure antioxidant compound as antibiofilm which will show more certain relation of the activities.

CONCLUSION

In conclusion, all of the samples had antioxidant activity. The clove leaves and leaffruit plants had the highest antioxidant activities, while the bay leaves had the lowest antioxidant activity. For the antibiofilm assay, all of the methanol extracts had antibiofilm activity, except cashew nut shell extract against *E. coli*. Mangosteen peel extract in methanol had the highest antibiofilm activity against all bacteria. However, for the n-hexane extract, the antibiofilm activity only showed in leaffruit plants against *E. coli* and cashew nut shell against *S. enteritidis*. The plant extracts which showed high antioxidant and antibiofilm activities such as leaffruit plants, mangosteen peel and clove leaves might be applied as feed supplement for controlling pathogenic bacteria.

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Bioactive Substances of Some Herbals and Their Effectiveness as Antioxidant, Antibacteria and Antifungi

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ABSTRAK

Sinurat AP, Wina E, Rakhmani SIW, Wardhani T, Haryati T, Purwadaria T. 2018. Zat bioaktif dari beberapa tanaman herbal dan keefektifannya sebagai antioksidan, antibakteri dan antijamur. JITV 23(1): 18-27. DOI: http://dx.doi.org/10.14334/jitv.v23i1.1660

Penelitian dilakukan untuk mengetahui zat bioaktif dari beberapa tanaman lokal dan keefektifannya sebagai antioksidan, antibakteri dan antiamur untuk digunakan sebagai imbuhan pakan, Sebanyak 12 tanaman digunakan dalam penelitian ini. Pengujian dilakukan terhadap kandungan total fenol, tanin dan saponin dalam ekstrak tanaman. Ekstrak tanaman juga diuji aktifitas antioksidan dan kemampuan untuk menekan produksi gas mikroba dari usus ayam secara in vitro, kemampuan untuk menghambat pertumbuhan bakteri (E.coli, S. enteritidis) dan jamur (A. niger). Hasil penelitian menunjukkan bahwa kandungan total fenol tanin tertinggi diperoleh pada ekstrak daun cengkeh, sementara kandungan saponin tetinggi diperoleh pada ekstrak daging buah Sapindus rarak. Aktifitas antioksidan tertinggi terdapat pada ekstrak daun meniran. Gas yang diproduksi oleh mikroba menurun seperti daya kerja antibiotik, dengan menambahkan pakan dengan daun meniran yang diekstraksi dengan hexan, biji kapok atau kulit buah manggis atau daun cengkeh yang diekstraksi dengan metanol. Efek inhibitor terhadap jamur diperoleh dari ekstrak daun cengkeh. Efek penghambat terbaik terhadap pertumbuhan E. coli (diukur dengan zona terang), ditemukan pada ekstrak metanol daging buah S. rarak. Penghambat yang paling efektif terhadap pertumbuhan E. coli dan salmonella adalah asap cair kulit kacang mete. Ekstrak daun cengkeh (antijamur), asap cair kulit kacang mete (antibakteri), daun meniran (antioksidan) mempunyai potensi untuk digunakan sebagai imbuhan pakan sebagai pengganti antibiotic-growth promoter.

Kata Kunci: Zat Bioaktif, Herbal, Antioksidan, Antibakteri, Antijamur

ABSTRACT

Sinurat AP, Wina E, Rakhmani SIW, Wardhani T, Haryati T, Purwadaria T. 2018. Bioactive substances of some herbals and their effectiveness as antioxidant, antibacteria and antifungi. JITV 23(1): 18-27. DOI: http://dx.doi.org/10.14334/jitv.v23i1.1660

A study was conducted to explore the bioactive substances of some local plants in order to find their effectiveness as antioxidant, antibacteria and antifungi to be used as feed additives. Twelve plants material were used in this study. The total phenol, tannin and saponin contents in the plant extract were assayed. The extracts were also assayed on their antioxidant activities and on their ability to depress in vitro gas production of microbes obtained from chicken's guts, their ability to inhibit growth of bacteria (E. coli and Salmonella enteridis) and fungi (A. niger). The results showed that the highest total phenol and total tannin contents were found in clove leaf extract, while the highest saponin content was found in Sapindus rarak fruit pericarp. The highest antioxidant activity was found in the leaffruit extract. Gas produced by microorganims was reduced to the level similar to antibiotic addition were found with addition of hexane- extract of leaffruit, kapok seed or methanol- extract of mangosteen fruit rind pulp or clove leaves. The best inhibitory effect on E. coli growth (measured by clearing zone) was found in methanol extract of S. rarak fruit. However, the most effective growth inhibitor for both E. coli and Salmonella was the liquid smoke of cashew nut shell. The best growth inhibitor for fungal growth was found in extract of clove leaves. Therefore, clove leaves extract (anti fungi), liquid smoke of cashew nut shell (antibacteria) and leaffruit (antioxidant) may have potential to produce feed additives to substitute antibiotic growth promoters.

Key Words: Bioactive Substances, Herbals, Antioxidant, Antibacteria, Antifungi

INTRODUCTION

Feed additives are commonly used for animal production in aim to improve the productive performance, feed utilization efficiency and quality of animal products. One of the feed additives that widely used is antibiotic, known as antibiotic growth promoters (AGP). The AGP has been used since 1940's (Dibner & Richards 2005) and legally recognized as feed additives in all over the world until 1970's when the Swann Reports 1969 showed the negative impact of the AGP usage such as the occurrence of microorganism resistant to antibiotics.

Since the Swann report, many countries banned or at least restricted the use of AGP. Indonesia also bans the use of AGP in animal feed as stated in the

legislation Act 18/2009 juncto Act 41/2014. Endorsement of the regulation without precaution may deteriorate the poultry productivity and feed efficiency drastically. Therefore, it is important to find non-antibiotic feed additives to substitute for the AGP. On the other hand, many reports and opinion conclude that the use of AGP promote the occurrence of antibiotic resistant microorganisms which is harmful for the human.

Some efforts have been done in search of materials to replace the AGP which are "friendly" for environments and human health. Among them are enzymes, acidifiers, probiotic, prebiotic, plant bioactives and the combination of those products. As a tropical country, Indonesia has abundant of plants as source of plant bioactives. Some plants or herbs have been used traditionally as "healthy drinks" and for medications for human in Indonesia and elsewhere.

Bioactive compounds in plants are defined as compounds produced by plants having pharmacological or toxicological effects in man and animals (Bernhoft 2010). The typical bioactive compounds in plants are produced as secondary metabolites. Plant bioactives have been reported to have functions as antibiotic, anti fungi, antioxidant and immuno modulator. Some researches have been conducted in order to proof the beneficial effect of plant bioactives to replace AGP such as Aloe vera (Purwadaria et al. 2001; Bintang et al. 2001; Sinurat et al. 2003; Sinurat et al. 2004; Sinurat 2013), Curcuma longa and Curcuma xanthorrhiza (Samarasinghe et al. 2003; Sinurat et al. 2009), and guava leaves (Hoque et al. 2007; Kidaha et al. 2013). Most of the researches studied the effectivity of the bioactives obtained from single ingredient and the results have not been applied in the livestock industry commercially due to some factors such: variability on the effectivity, high production cost or too expensive when compared to the commercial AGP and when the use of AGP is still allowed in the country.

Synthetic antioxidants such as ethoxyquin, BHA (butylated hydroxyanisole) and BHT (butylated hydroxytoluene) are also used as feed additives in order to improve the healthyness (immunity of the animals). The plant antioxidant can induce the phase 2 enzymes or cytoprotective proteins in the cell and act to neutralize toxic agents when they appear (Blomhoff 2010). Farahat et al. (2016) reported that natural antioxidant obtained from decaffeinated green tea extract are effectively to enhance the immune response and vaccination potency in broilers without adversely affected the growth performance of broilers. The addition of antioxidant in feed may help the animal to 'fight' pathogen microorganisms.

In order to find an alternative to AGP, this research was focused on the searching of plants that contain bioactives effective for inhibiting bacteria growth, fungus growth and have high antioxidant activity. The combination of three bioactives obtained from plants are expected to form a feed additive that can replace or even better than antibiotic growth promoters.

MATERIALS AND METHODS

Source of plant bioactives

Twelve (12) plants which have been kown in literatures contain bioactives and have been used in society as traditional drinks or medications were used in this study, i.e.,: Binahong (*Anredera cordifolia*) leaves, clove (*Syzygium aromaticum*) leaves, guava (*Psidium guajava)* leaves, Mangosteen (*Garcinia mangostana)* fruit shell, Mimba (*Azadirachta indica*) leaves, Mindi (*Melia azedarach L*.) leaves, salam (*Syzygium polyanthum*) leaves, cashew nut shell, Sirih (*Piper betle*) leaves, meniran (*Phyllanthus urinaria)*, kapok seed oil and lerak (*Sapindus rarak*) fruits. The characteristics of the plant bioactives used in the experiment were:

1. Salam (*Syzygium polyanthum*) leaves with bioactive components such as antioxidant, antiinflammation, antiallergy (Kusuma et al. 2011).
2. Binahong (*Anredera cordifolia*) leaves contain flavonoids which have antibacterial activity (Astuti et al. 2011).
3. Mimba *(Azadirachta indica)* leaves contain bioactive that effective as antibacteria, particularly against *Vibrio cholerae* (Thakurta et al. 2007).
4. Lerak (*Sapindus rarak De Candole*) fruits pericarp with high saponin contents and effectively inhibit growth of *Escherichia coli* (Astuti et al. 2009) and *Eimeria tenella* (Pasaribu et al. 2014).
5. Kapok (*Ceiba pentandra*) seed oil with high contents on cyclopropanoids which are effective to inhibit growth of *Bacillus subtilis* (Chekuboyina et al. 2012).
6. Guava (*Psidium guajava*) leaves contain tannin, tripertene, flavonoid, and have been reported to be effective as antioxidant and antimicrobes (Biswas et al. 2013).
7. Clove (*Syzygium aromaticum*) leaves are rich in eugenol which are effective as anti-fungi such as *Fusarium oxysporum* and anti-bacteria such as *Pseudomonas solanacearum* (Pandey & Singh 2011).
8. Sirih (*Piper betel*) leaves contain hydroxychavicol, eugenol, which are effective as antiulcer, anthelmintic, antioxidant (Chakraborty & Shah 2011).
9. Mindi (*Melia azedarach* L.) leaves contain terpenoid and azadirachtin (Meziane and Goumri, 2015) which are effective as antibacteria, antivirus, insectiside and as antioxidant (Khan et al. 2011).

10. Mangosteen (*Garcinia mangostana*) fruit pericarp is rich in xanthones which are known as antioxidant, anti inflamation, antibacteria and antifungi (Palakawong et al. 2010). The ethanol extract have been reported effective to inhibit growth of pathogen bacteria that usually found in gastro intestinal tract such as *Salmonella typhi*, *Shigella dysentriae*, *E. coli*, *Klebsiella pneumoniae*, *V. cholerae*, *Pseudo-monas aeruginosa* and *Staphylococcus aureus* (Geetha et al. 2011).
11. Liquid smoke obtained from Cashew nut (*Anacardium occidetale*) shell contains anacardic acids (aphenolic lipids) which are effective as antifungal and anti-microbial (Parasa et al. 2011). The liquid smoke has a low pH and contains high phenolic compounds which may be effective to be used as an antimicrobial in feed (Saenab et al. 2016).
12. Gripeweed or leaffruit (*Phyllanthus urinaria*) plants commonly called chamber bitter or leafflower, is a herb species in the family Phyllanthaceae. The bioactives were reported as high antioxidant activities and effective as antibacteria (Kumaran & Karanukaran 2007; Eldeen et al. 2010).

Preparation of plant crude extract

Samples of plant materials were dried and ground prior to extract. The samples were extracted by methanol for polar fraction or by hexane for non-polar fraction. Each 5 g of the sample was soaked in 10 ml methanol solution. The soaking was performed with sonicator for 30 minutes. The solutions were then centrifuged for 15 minutes (2500 rpm) and the supernatant was collected as a polar fraction.

Similar procedures were performed to obtain the extraction of the non-polar fraction by using hexane instead of methanol.

Total phenol and tannin

Phenol levels in the samples were measured by Folin-Ciocalteu method. Analyses were performed twice, i.e. before and after addition of polyvinyl polypyrrolidone (PVPP). The methods have been described by Makkar (2013)

Analysis of antioxidant potential

The antioxidant content of plant extraxts were determined following method as described by Shekar & Anju (2014) The procedure in brief was as follows: 0.5 g of dried samples were diluted with 10 mL methanol. The solution (1 mL) were made in some different concentrations and each was reacted with 2 mL 2,2 diphenyl-1-1-picrylhydrazil (DPPH) 0.2 mM. Then, they were incubated at room temperatures for 30 minutes. The assay was performed in dark room and the absorbance of the solution was measured at 517 nm. Methanol was used as the blank (1 mL methanol added with 2 mL DPPH 0.2 mM). Concentration to inhibit 50% oxidation, ng/mL was calculated in each curve of extract concentration towards oxidative inhibitions.

$$\% \text{ DPPH inhibition} = \frac{A - B}{B} \times 100\%$$

A = Absorbance of control
B = Absorbance of sample

Effectiveness to inhibit microorganism growth

Two methods were performed in order to identify the effectiveness of the plant bioactives to inhibit the growth of microorganisms. The first method was the in vitro fermentation method or by measuring the total gas production. In this method, 0.1 g pepsin digested corn meal as substrate in the 15 ml glass test tube, was added with 0.5 ml plant extract and 10 mL artificial saliva buffer (McDougall buffer) which has been mixed with the **c**hicken digesta as inoculum with ratio 4:1 to mimic gut's condition during fermentation.

The CO_2 gas was ran for 15-20 minutes into the digesta-buffer prior to addition of the samples. After mixed homogenously and flushed with the CO_2 gas, the tubes were tightly sealed and incubated at 37-40ºC for 24 hours. Samples without plant extract were performed as negative control. The gas produced was measured every hour till 9 hours of fermentation and at the end of fermentation (24h). Total gas production was calculated as the sum of gas produced during 24 h of incubation. The amount difference between total gas production in the treated tubes and the control (without plant extract) were considered as the indicator on the effectiveness of the extract to inhibit microorganism growth. The higher the difference (or the less total gas production), the more effective the plant extract to inhibit the microorganism growth. Each treatment was replicated 4 (four) times.

The second method was by observing the growth of *E. coli* and *Salmonella enteritidis* in petri dish as described by Rollins & Joseph (2000). Assays were performed by placing paper on the agar media inoculated with *E. coli* or *S. enteritidis sp*. The diameter of *clear zone* was measured as an indicator of effectiveness of the plant extract to inhibit the bacteria growth.

Substrate preparation and source of microbes for inoculum

Substrate was prepared as follows: 10 g corn meal was mixed with 300 ml pepsin solution (0.2% in HCl

0.1 N). The mixture was incubated on water bath shaker at 40°C for 45 minutes followed by centrifugation at 3000 rpm for 10 minutes. Then, the precipitate was washed with distilled water, and again centrifuged at 3000 rpm for 5 minutes, dried and regarded as substrate for in vitro fermentation.

Source of microbes used for in vitro fermentation in this experiment was total microbes that present in the digesta of the chickens and were attacked by antibiotic growth promotors. The digesta were collected by squeezing the contents of the guts of chickens obtained from poultry slaughter house. The digesta were pooled and kept in the freezer and used as inoculum for in vitro fermentation.

RESULTS AND DISCUSSION

Identification of bioactive substances of some native plants

Concentration of some bioactive compounds in some plants extract is shown in Table 1. Extract of clove leaves contain the highest total phenol (6.04 %) followed by the mangosteen fruit pericarp extract (4.76 %) as compared to other plant extracts. The kapok seed oil contained the lowest (not detected) total phenol. Similar to the total phenol content, the highest tannin levels was also found in clove leaf extract (3.34%) followed by mangosteen fruit pericarp extract (2.72%) and the lowest (not detected) in kapok seed oil. The tannin level in both clove leaf and mangosteen extract were found more than 50% of the total phenol. The saponin level, however, showed different trend. The highest saponin level was detected in *S. rarak* fruit pericarp extract (59.71%) followed by liquid smoke of cashew nut shell (50.46%) and the lowest level was found in kapok seed oil (1.19 %). Total phenol, tannin and saponin are compounds that dissolved in polar solvents, therefore their content in kapok seed oil which dissolved in non polar solvent was very low compared to other plant extracts in this study.

Plant phenolic compounds and tannins are well known as potent antioxidant (Ghasemzadeh & Ghasemzadeh 2011) and can serve as protectants against bacterial pathogen (Sytar et al. 2012). Plant saponin was also reported to inhibit sporulation of *E. tenella* oocysts (Pasaribu et al. 2011). Therefore, it is expected that the highest the content of the total phenol, tannin and saponin in the plants the higher the potential benefit for the consumers (animals).

Analyses on antioxidant activities of the samples were conducted in comparison with vitamin C (as standard) and the concentrations of plant extracts that effectively inhibit 50% oxidation are shown in Table 2. The leaffruit *(Phyllanthus urinaria)* extract showed the least concentration (124 µg/mL) followed by clove leaves extract (147.9 µg/mL) and cashew nut liquid smoke (156.5 µg/mL). The antioxidant activity of

Table 1. Concentrations of total phenol, tannin and saponin in some plant extracts

Plants name	Parts	Method of Process	Total Phenol (% DM sample)	Tannin (%)	Saponin (%)
Binahong (*A. cordifolia)*	Leaves	Extraction	0.48	0.16	4.94
Clove (*S. aromaticum)*	Leaves	Extraction	6.04	3.34	13.50
Guava (*P. guajava)*	Leaves	Extraction	3.34	2.30	5.70
Lerak (*S. rarak*)	Fruit pericarp	Extraction	0.24	0.05	59.71
Mangosteen (*G. mangostana)*	Fruit fruit pericarp	Extraction	4.76	2.72	15.23
Leaffruit (*P. urinaria)*	Whole plant	Extraction	3.06	1.67	2.14
Cashew nut (*A. occidetale)*	Shell	Liquid smoke	2.79*	1.42*	50.46*
Neem (*A. indica*)	Leaves	Extraction	2.24	0.38	13.39
Mindi (*M. azedarach*	Leaves	Extraction	0.94	0.34	16.29
Kapok (*C. pentandra)*	Seed oil	Screw pressed	ND	ND	1.19
Salam (*S. polyanthum)*	Leaves	Extraction	1.45	0.36	13.50
Sirih or betel plant (*P. betle)*	Leaves	Extraction	3.18	2.30	6.88

* Percentage in 100 ml liquid smoke

Table 2. Antioxidant activity of some plant extracts

Plants name	Parts	Concentration to inhibit 50% oxidation, ng/mL
Binahong (*A. cordifolia)**	Leaves	ND
Clove (*S. aromaticum)*	Leaves	274.67
Guava (*P. guajava)*	Leaves	625.95
Lerak (*S. rarak)*	Fruit pericarp	6995.66
Mangosteen (*G. mangostana)*	Fruit pericarp	338.42
Leaffruit (*P. urinaria)*	Whole plant	292.08
Cashew nut (*A. occidetale)*	Shell	359.58
Neem (*A. indica*)	Leaves	555.66
Mindi (*M. azedarach*	Leaves	NA
Kapok (*C. pentandra)*	Seed oil	NA
Salam (*S. polyanthum)*	Leaves	759.72
Sirih or betel plant (*P. betel)*	Leaves	532.52

*The antioxidant activities of some plants were also correlated with antibiofilm activity (Gracia et al. 2017);
ND = not detected or no antioxidant activity; NA = not analysed

Binahong (*A. cordifolia)* leave extract, Mindi (*M. azedarach)* and Kapok (*C. pentandra)* seed oil could not be obtained since the green colour of binahong and mindi extract disturbed the color measurement during the analysis, while oil fraction of Kapok seed oil could not mix with the DPPH solution in the analysis. The least the concentration required to inhibit oxidation means the more effective the substances as antioxidant. Therefore, the leaffruit *(P. urinaria)* extract and clove extract showed the best potential antioxidant activity.

Plant phenols are antioxidants by virtue of the hydrogen-donating properties of the phenolic hydroxyl groups (Blomhoff 2010). However, the results showed that the total phenol was not directly related to the antioxidant activity. As shown in Table 1, the highest total phenol was found in clove leaves extract (6.04 %), while the highest antioxidant activity was found in leaffruit extract (Table 2). Although, in general, samples with high antioxidant activities (leaffruit, clove leaves, and mangosteen fruit rind pulp) always contain high total phenols. The phenols have many sturctural component such as phenolic acids, flavonoids, stilbenes and lignans. The effectivity of each compound as antioxidant may be not the same and the level of each compound may different in different plants.

The effectiveness of the plant extracts to inhibit growth of microorganisms (in-vitro) was determined by volume of gas produced by microorganisms obtained from gastro intestinal tract of chickens during 24 h. As shown in Table 3 the addition of antibiotic (50 ppm zinc bacitracin) or the plant extracts significantly ($P<0.05$) affected the amount of gas produced during the incubation. The gas production was significantly reduced by addition of the antibiotic (50 ppm zinc bacitracin) to 65% as compared to the control without antibiotic. Reduction of gas production to the level similar to antibiotic were found with addition of hexane extract of leaffruit (72% of control), kapok seed oil (69% of control), and methanol extract of mangosteen fruit pericarp (61.0% of control), clove leaves (63% of control), *S. polyanthum* leaves (72% of control), guava leaves (74% of control) and *M. azadirach* leaves (77% of control). If the reduction of total gas production can be used as indicator of the effectiveness to inhibit pathogen microorganisms, it could be concluded that these plant extracts are the best candidates to replace antibiotic. However, gas production in this method is the result of all microorganisms (obtained from chicken intestinal) including pathogens and non-pathogens. On the other hand, the purpose of this research was to explore the effectiveness of plant bioactives to inhibit growth of pathogen microorganisms which may be used as alternatives to AGP. Perhaps, gas production may only be used as a preliminary tool to indicate that the plant extract contains substances as anti-microorganism. The conventional method (diffusion test) by measuring the size of clear zone arround the samples place on an agar plate may still the best method to measure the effectiveness of a substance as an antibiotic.

Results of diffusion method to test the effectiveness of the plant extracts to inhibit growth of *E. coli* and *S. enteritidis* is presented in Table 4. Negative control did not have a clear zone diameter (0 mm), but addition of antibiotic (pyripen 5.5 mg/10ml) produced

Table 3. In-vitro gas production of digested corn by microbes (obtained from chickens gastrointestinal tract) for 24 h as affected by plant extract addition

Plants' name	Hexane fraction (50 mg/ml extract)		Methanol fraction (50 mg/ml extract)	
	Total gas production, mL/ 24 h*	Relative to Control	Total gas production, mL/ 24 h*	Relative to Control
Binahong (*A. cordifolia*)	24.63^{a}	0.95	20.35^{abc}	0.88
Clove (*S. aromaticum*)	24.98^{a}	0.97	14.60^{d}	0.63
Guava (*P. guajava*)	22.83^{ab}	0.88	17.08^{bcd}	0.74
Lerak (*S. rarak*)	26.05^{a}	1.01	22.38^{ab}	0.97
Mangosteen (*G.mangostana*)	22.67^{ab}	0.88	14.20^{d}	0.61
Leaffruit (*P. urinaria*)	18.7^{bc}	0.72	ND	
Cashew nut (*A. occidetale*) shell	23.1^{ab}	0.89	17.5^{bcd}	0.75
Neem (*A. indica*)	24.05^{a}	0.93	18.57^{abcd}	0.80
Mindi (*M. azedarach*)	24.83^{a}	0.96	17.70^{bcd}	0.77
Kapok (*C. pentandra*)	17.84^{bc}	0.69	20.60^{ab}c	0.89
Salam (*S. polyanthum*)	21.83^{abc}	0.84	16.73^{cd}	0.72
Sirih or betel plant (*P. betle*)	23.63^{a}	0.91	18.75^{acd}	0.81
Control (C)	25.88^{a}	1.00	23.13^{a}	1.00
C + Zinc Bacitracin	16.68^{c}	0.65	16.68^{c}	0.65

* Different letters in superscripts showed significant different ($p<0.05$); ND = Not determined; Control was not added with plant extracts.

significantly clear zone diameter. Not all the extracts produced clear zone (or ability to inhibit growth of *E. coli* or *S. enteritidis*). Almost all the methanol extract showed inhibition of E. coli with different clear zone diameters except extracts of betel plant (*P. betel)* leaves, leaffruit (*P. urinaria),* Salam (*S. polyanthum)* and Neem (*A. indica*) leaves. However, only the hexane extraxt of kapok seed and *S. rarak* showed positive effect to inhibit *E. coli*. The most effective to inhibit *E. coli*, other than antibiotic was the methanol extract of *S. rarak* fruit (2.05 mm) followed by methanol extract of clove leaves (1.98 mm), methanol extract of cashew nut shell (1.90 mm) and liquid smoke of cashew nut shell (1.2 mm). The hexane extract showed only kapok seed oil and *S. rarak* fruit pericarp were slightly effective to inhibit *E. coli* growth, but hexane extract of mangosteen fruit pericarp, kapok seed, *S. rarak* fruit pericarp, guava leaves, *A. cordifolia* leaves, leaffruit, neem slightly inhibited the growth of *S. enteritidis*. In terms of effectiveness, methanol extracts which contain phenolic compounds and other polar bioactive compounds were more effective than hexane extracts to inhibit the growth of *E. coli*, while hexane extract which contain nonpolar bioactive compounds were more effective than methanol extracts to inhibit the growth of *S. enteritidis*. Both bacteria are Gram-negative and always found in digestive tracts, however, every species including their strains have been recognised differently sensitivity or resistency to antibacteria especially for those not included in broadspectrum. Some of *E. coli* strains and most *S. enteritidis* are pathogens. The bacteria used in this experiment was isolated from chicken digestive tract and pathogen, therefore the antibacteria activity detected in the plant extracts may prove the idea to replace the AGP with the plant extracts.

Inclusion of zinc bacitracin in the diet alter the microbiota composition in the ileum of broilers (Gong et al. 2008). Similar result was also reported by Engberg et al. (2000) which showed that addition of salinomycin or zinc bacitracin reduced the population of *Clostridium perfringens* and *Lactobacillus salivarius* in the intstinal of broilers.

Addition of anti-fungi (ketoconazole) showed effectively inhibit the growth of *A. niger* with the clear zone diameter 1.13 – 2.63 cm. Almost all plant extracts tested were not effective to inhibit growth of fungi (*A. niger* BPT) as shown in Table 5. However, extract of Clove (*S. aromaticum)* leaves and liquid smoke of Cashew nut (*A. occidetale)* shell were effective to inhibit the growth of the fungi. The clove leave meal extract showed the highest anti fungi activities with diameter of clear zone 1.24 cm and the liquid smoke of cashew nut shell with diameter of clear zone 1.18 cm.

Table 4. Diameter (mm) of clear area on the culture of *E. coli* and *S. enteritidis* as affected by supplementation of plants extract

Source of extracts	Methanol extract		Hexane extract	
	E. coli	*S. enteritidis*	*E. coli*	*S. enteritidis*
Negative control	0^{f}	0^{c}	0	0
Positive control*	3.16^{a}	3.05^{a}	2.28	2.9
Mangosteen (*G. mangostana)*	1.77^{abc}	0^{c}	-	1.1
Betel plant (*Piper betel)*	0^{f}	0^{c}	-	-
Clove (*S. aromaticum)*	1.98^{ab}	0^{c}	-	-
Kapok (*C. pentandra)*	1.63^{bcd}	0^{c}	1.13	1.05
Lerak (*S. rarak)*	2.05^{ab}	0^{c}	1.1	1.1
Guava (*P. guajava)*	1.23^{e}	0^{c}	-	1.1
Mindi (*M. azedarach*)	1.20^{e}	0^{c}	-	-
Cashew nut (*A. occidetale)* Shell hexane extract	1.90^{ab}	0^{c}	-	-
Binahong (*A. cordifolia)*	1.40^{e}	0^{c}	-	1.1
Leaffruit (*P. urinaria)*	0^{f}	0^{c}	-	1.12
Salam (*S. polyanthum)*	0^{f}	0^{c}	-	-
Neem (*A. indica*)	0^{f}	0^{c}	-	1.1
Liquid smoke of Cashew nut (*A. occidetale)* shell	1.2^{ed}	1.15^{b}	1.2^{ed}	1.15^{b}
Significance (P)	<0.001	<0.001		

* antibiotic (pyripen 5.5 µg/10ml) was added

Table 5. Diameter (cm) of clear area on the culture of fungi (A. *niger* BPT) as affected by supplementation of plants extract

Source of extract	Diameter of clear zone (cm)	
	Control + Ketoconazole	*A. niger* BPT
Leaffruit (*P. urinaria)* plants	1.13	0.00
Clove (*S. aromaticum)* leaves	2.63	1.24
Neem (*A. indica*) leaves	1.40	0.00
Lerak (*S. rarak)* fruit	1.27	0.00
Liquid smoke of Cashew nut (*A. occidetale)* shell	1.23	1.18
Binahong (*A. cordifolia)* leaves	1.20	0.00
Mindi (*M. azedarach)* leaves	1.23	0.00
Kapok (*C. pentandra)* seed oil	1.13	0.00
Betel plant (*P. betel)* leaves	1.13	0.00
Salam (*S. polyanthum)* leaves	1.13	0.00
Mangosteen (*G. mangostana)*	1.17	0.00
Guava (*P. guajava)*	1.33	0.00

The bioactive compounds in cloves leaf extract or oil are well known as eugenol (Yitbarek 2015). The eugenol has been used for medication purposes in human. Joseph & Sujatha (2011) reported that the clove crude extract and the clove oil were effective to inhibit of some fungi such as *Paeciliomyces, A. flavus, A. niger, Penicillium* sp., *Rhizopus* sp. and *Rhizomucor* sp. Liquid smoke of cashew nut shell has low pH and contain many simple phenols (Saenab et al. 2016) that have the ability to depress the growth of bacteria and fungi.

CONCLUSION

Bioactive components such as total phenol, tannin and saponin of plant extract could not be used as an indicator of their effectiveness as antioxidant, antibacterial or antifungi, neither the ability to reduce the in vitro total gas production of microbes obtained from chicken's gut. The conventional method carried out to measure antioxidant activity, growth inhibition (of *E. coli* and *S. enteritidis*), and growth inhibition of fungi (*A. niger*) concluded that leaffruit extract, the liquid smoke of cashew nut shell and the clove leaves extract performed the highest potential as antioxidant, antibiotic and anti-fungi, respectively. Further study is required to prove if they could be used to substitute antibiotic growth promoters in feed.

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Substitution of Mulberry Leaf Meal on Feed Intake, Body Weight and Carcass Characteristics of Tigray Highland Lambs

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ABSTRAK

Tesfay G, Tamir B, Berhane G. 2018. Substitusi tepung daun *mulberry* terhadap konsumsi pakan, bobot badan dan karakteristik karkas anak domba dataran tinggi Tigray. JITV 23(1): 28-37. DOI: http://dx.doi.org/10.14334/jitv.v23i1.1634

Tujuan dari penelitian ini adalah untuk mengevaluasi pengaruh substitusi tepung daun *mulberry* sebagian maupun penuh sebagai campuran konsentrat terhadap performa anak domba dataran tinggi Tigray. Sebanyak tiga puluh ekor anak domba jantan dataran tinggi Tigray berumur satu tahun (rata-rata bobot badan 17,8±0.95 kg) dikelompokkan ke dalam 6 kelompok berdasarkan bobot hidup yang diberikan 5 jenis perlakuan pakan (RCBD), yaitu T1: 300 g campuran konsentrat tunggal; T2: 225 g campuran konsentrat + 86,55 g daun *mulberry*; T3: 150 g campuran konsentrat + 173,1 g daun *mulberry*; T4: 75 g campuran konsentrat + 259,7 g daun *mulberry*; dan T5: 346,2 g daun *mulberry* tunggal. Perlakuan pakan tersebut dikondisikan sebagaimana mungkin untuk menggantikan campuran konsentrat dengan tepung daun *mulberry* secara perlahan dari 0% hingga 100% dalam kondisi iso-nitrogenous. Anak domba diadaptasikan pada pakan percobaan selama 15 hari dan setelah itu dilakukan percobaan pemberian pakan. Hasil pengamatan menunjukkan bahwa penggantian penuh campuran konsentrat dengan tepung daun *mulberry* memiliki nilai total konsumsi bahan kering, bahan organik, NDF dan ADF yang lebih tinggi (P<0,05) dibandingkan dengan pemberian campuran konsentrat tunggal. Parameter performa pertumbuhan sebanding pada semua perlakuan pakan. Bobot potong dan bobot tanpa jeroan pada pemberian pakan tepung daun *mulberry* tunggal lebih besar (P<0,05) dibandingkan dengan perlakuan pemberian campuran konsentrat secara penuh. Dengan kata lain, persentase karkas terhadap bobot badan tanpa jeroan dasar dan bobot carcas panas tidak berbeda (P>0,05) pada perlakuan yang berbeda. Oleh karena itu, daun *mulberry* dapat berpotensi digunakan untuk penggantian campuran konsentrat sebagai suplemen pakan untuk peternak skala kecil di Ethiopia.

Kata Kunci: Daun *Mulberry*, Daya Cerna, Pertambahan Bobot Badan

ABSTRACT

Tesfay G, Tamir B, Berhane G. 2018. Substitution of mulberry leaf meal on feed intake, body weight and carcass characteristics of Tigray highland lambs. JITV 23(1): 28-37. DOI: http://dx.doi.org/10.14334/jitv.v23i1.1634

The purpose of this study is to evaluate effects of partial or full substitution of mulberry leaf meal for concentrate mix on performances of Tigray highland lambs. Thirty intact yearlings Tigray highland male lambs (average initial body weights of 17.8±0.95 kg) were separated into 6 groups based on their live weight with each groups assigned 5 treatment diets (RCBD), that are: T1: 300 g concentrate mix alone, T2: 225 g concentrate mix + 86.55 g mulberry leaf, T3: 150 g concentrate mix + 173.1 g mulberry leaf, T4: 75 g concentrate mix + 259.7 g mulberry leaf and T5: 346.2 g mulberry leaf alone. The treatments diets were designed in such a way that concentrate mix was progressively replaced by mulberry leaf meal from 0% to 100% at iso-nitrogenous level. Lambs were adapted to experimental diets for 15 days, and after adaptation period, feeding trial was conducted. Results reveal that complete substitution of concentrate mix by mulberry leaf meal showed in higher (P<0.05) total dry matter, organic matter, NDF and ADF intake than the sole concentrate mix. The growth performance parameters resulted comparable across all the treatment diets. The slaughter weight and empty weight resulted higher (P<0.05) in sole mulberry leaf meal as compared to the whole concentrate mix supplemented lambs. On the other hand, the dressing percentage on empty body weight base and hot carcass weight showed less difference (P>0.05) across the different treatments. Therefore, mulberry foliage could potentially be used to replace concentrate mix as a feed supplement for the small holder farmers in Ethiopia.

Key Words: Mulberry Leaf, Digestibily, Weight Gain

INTRODUCTION

In Ethiopia sheep accounted 34% of the live animal exports (Gizaw et al. 2013). Moreover, sheep and goats contributed 86% of the total value of meat exports (Legese & Fadiga 2014). Even though the sheep population provided considerable roles to both smallholder farmers and the country's economy but their present contribution is far below their potential. This is due to the quantity and quality of feed resources

available and feeding systems employed which have great impact on their production and productivity. In areas where livestock are closely integrated with crop production, crop residues are considered as valuable sources of ruminant feeds. The tendency of increased acreage of cropping land is always at the expenses of decreased available grazing lands, thus boost the importance of crop residues as animal feed resources. However, crop residues are of generally low in nutritive value and are fibrous having low digestible organic matter (OM) per kg dry matter (DM)) and low crude protein (CP) content (Tolera 2008); Gizaw et al. 2010).

The increasing pressure on land and the growing demand for livestock products makes it crucial to ensure the effective use of feed resources, including crop residues and other agro-industrial by-products. With the increasing need of human population for animal products, there is a need of matching feed resources available with animal nutrient requirements. Thus, supplementation of crop residues with agro-industrial by-products and/or plant protein sources will alleviate CP deficiency in fibrous feeds (Solomon 2001).

The commercial protein supplements are however, inaccessible and if any too expensive for the small scale farmers. Different studies indicated that mulberry is well comparable with leguminous multipurpose trees and concentrates as a feed for ruminants (Benavides et al. 2000; Sanchez 2002; Cuong et al. 2007; Kabi & Bareeba 2008; Kandylis et al. 2009). Habib et al. (2016) evaluated the chemical composition of dried mulberry leaves and reported that it has comparable nutrient composition with that of commercial concentrates diets. However, the information on the feeding value of mulberry foliage and its concomitant effect on feed intake, weight gain and carcass characteristics of lambs in Ethiopia are scanty and need further documentation. To this end (FAO 2002) suggested that the urgent need of the farmers for high quality feed for ruminants in developing countries can be achievable through intensive utilization of multipurpose trees and shrubs as they have better nutritional quality nearly equivalent to grain based concentrates.

According to Shayo (1998), leaves of multipurpose trees like mulberry are highly digestible (82%); contain high concentration of CP (18.6%) and mineral matters (14.3), and low cell wall contents. This important characteristic of mulberry foliage is a key attribute that makes it worthy of investigation as feed alternative for ruminants. Nevertheless, there are very limited research reports on the use of mulberry for small ruminants in Ethiopia. Therefore, this study was carried out with the objective of investigating the effect of partial or full substitution of mulberry foliage for concentrate mix on performance of yearling Tigray highland sheep fed barley straw basal diet.

MATERIALS AND METHODS

Experimental animals, experimental design, treatments and feed intake

Thirty intact yearlings Tigray highland male lambs with average initial body weights of 17.8±0.95 kg having relatively similar body condition were used for the study. The sheep were quarantined, drenched against internal parasites, sprayed for ecto-parasites and vaccinated for anthrax as well as ovine pasteurellosis. They were adapted to experimental feeds for 15 days followed by 90 days of feeding period to determine effect on feed intake and carcass parameters. The supplements were offered in two equal portions at 08:00 and 16:00 hours daily while the water and mineral salt licks were accessed freely to all lambs.

A complete randomized blocked design with five treatments of six animals each was employed and sheep were blocked on the basis of their initial body weight (overnight fasting) and the five treatments were randomly assigned to animals in the block. Lambs were housed in concrete floor that penned individually. The sheep were allocated to the diet groups where concentrate mix was progressively replaced by mulberry leaf meal from 0% to 100% at iso-nitrogenous level. The treatments were 300 g concentrate mix alone, 225 g concentrate mix + 86.55 g mulberry leaf, 150 g concentrate mix + 173.1 g mulberry leaf, 75 g concentrate mix + 259.7 g mulberry leaf and 346.2 g mulberry leaf alone with barley straw *ad libitum* to all treatments. The concentrate mix contained noug seed cake (NSC) and wheat bran (WB) at ratio of 1:2. Leaves from white mulberry (*Morus alba*) that was harvested from nearby farmers and nursery areas was collected and dried under a shade for 4-5 days till the leaves were easily crushed when pressed in a hand and was packed in a sack for later use. Feeds offered to experimental sheep and corresponding refusals were weighed and recorded daily throughout the experimental period to determine daily feed intake of experimental sheep. Samples of refusals collected from individual animals every day were pooled over the entire experimental period and sub-sampled for chemical analysis. Experimental animals were weighed on the first day of the feeding trial and subsequently at weekly intervals before offering the morning feed on the same day of the week after withholding feed and water overnight. The average daily body weight gain during the experimental period was calculated by regressing body weight of each animal on number of feeding days.

Table 1. Chemical composition of feeds used for the experiment

Experimental feeds	DM (%)	Chemical composition (%DM)							
		OM	Ash	CP	NDF	ADF	ADL	EE	CF
Barley straw	96.0	92.5	7.5	4.0	78.6	49.6	8.6	1.3	57.6
Concentrate mix	90.2	92.3	7.66	22.0	35.2	20.2	3.39	7.16	13.3
Mulberry leaf meal	91.8	84.3	15.7	18.5	38.0	22.3	4.19	4.15	14.2

DM: Dry matter
OM: Organic matter
CP: Crude protein
NDF: Neutral detergent fiber
ADF: Acid detergent fiber
ADL: Acid detergent lignin
EE: Ether Extract
CF: Crude fiber

Digestibility trial

After completion of 90 days of feeding trial, all animals were fitted with feces collection bag for in vivo digestibility test. The sheep were assigned to the same treatment diet they were offered in the feeding trial. After allowing the animals an adjustment period of three days to the feces collection harness, feces were collected for seven days. Each day's fecal output was weighed and 25% of it was frozen (−20°C) in containers meant for individual sheep and the seven days collection were pooled. The same was done for the feed offered and refusal samples collected. Composite samples of feed offered and refused and feces excreted were thawed to room temperature, mixed thoroughly and oven dried at 60 °C overnight. The dried samples of feeds and feces were ground to pass through 1 mm sieve and stored in airtight polyethylene containers until analyzed. Nutrient digestibility (%) was calculated as a difference between nutrient intake and nutrient voided in the feces divided by nutrient intake and the quotient multiplied by 100.

Chemical analysis of feed offered and refused

Chemical compositions of the feed and refusal samples were determined at Holeta Agricultural Research Center, Animal Nutrition Laboratory, Ethiopia. Samples of partially dried feeds and refusals were dried overnight at 105°C in a forced draft oven for determination of total dry matter content. Samples were analyzed for DM, ash, and nitrogen (N) using the procedure of AOAC (1990) and crude protein (CP) was calculated as N x 6.25. Neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL) were analyzed using the procedures of Van Soest et al. (1991). According to AFRC (1993), ME = 0.16*DOMD, DOMD = (Feed OM-Faeces OM)/Feed DM, where DOMD is digestible organic matter in dry matter.

Carcass evaluation

At the end of feeding trial, all experimental sheep were slaughtered after overnight fasting for evaluation of carcass parameters. Empty body weight was calculated as slaughter weight less gut content. Hot carcass weight was estimated after removing weight of the head, skin, thoracic, abdominal and pelvic cavity contents, and the limbs. Dressing percentage was calculated as a ratio of hot carcass weight and slaughter weight or empty body weight basis. The rib eye muscle area is determined by measuring area of the *Longissimus dorsi* muscle exposed by cutting the carcass between the 12^{th} and 13^{th} ribs (O'Rourke et al. 2004).

Statistical analysis

Data from the experiment were subjected to the analysis of variance (ANOVA) in a randomized complete block design using the general linear model procedure of SAS (2008). Individual differences between means were tested using Tukey HSD test. In all the comparisons, the level of significance was set at $\alpha = 0.05$.

RESULTS AND DISCUSSION

Dry matter and nutrient intake

Results of mean daily dry matter and nutrient intake of different proportions of concentrate to mulberry leaf meal, sole concentrate mix as well as sole mulberry leaf meal fed to the experimental sheep are given in Table 2. All lambs had a complete consumption of the supplemented mulberry and concentrate mix offered. Increasing the substitution rate of concentrate mix by mulberry leaf meal improved ($P<0.05$) the dry matter intake of lambs.

Total dry matter intake declined ($P<0.05$) when the proportion of mulberry leaf meal in the diet was decreased, and was lowest when an all concentrate diet was fed. Complete substitution of concentrate mix by mulberry leaf meal resulted in higher ($P<0.05$) total dry matter intake than those supplemented with whole concentrate mixtures.

Table 2. Daily intakes of Tigray highland lambs supplemented with graded levels of mulberry leaf meal and concentrate mix

Intake	Treatments					SEM	SL
	T1	T2	T3	T4	T5		
Dry matter							
Barley straw (g/day)	539	548	539	551	566	12.6	0.248
CM (g/day)	300	225	150	75		-	-
MLM (g/day)	-	86.6	173	260	346	-	-
TDM (g/day)	839^{c}	859^{bc}	863^{bc}	886^{ab}	912^{a}	12.6	0.0002
Nutrients							
OM (g/day)	776^{b}	787^{ab}	783^{ab}	798^{ab}	815^{a}	11.7	0.0282
CP (g/day)	87.7	87.5	86.6	86.6	86.6	0.51	0.115
EE (g/day)	28.5^{a}	26.8^{b}	24.9^{c}	23.3^{d}	21.7^{e}	0.16	<.0001
CF (g/day)	350^{b}	358^{ab}	354^{ab}	364^{ab}	375^{a}	5.64	0.0131
NDF (g/day)	529^{c}	542^{bc}	543^{bc}	558^{ab}	576^{a}	6.78	0.0001
ADF (g/day)	328^{b}	336^{b}	336^{b}	347^{ab}	358^{a}	6.27	0.0014
ME (MJ/day)	8.51^{b}	8.77^{ab}	8.70^{ab}	8.97^{ab}	9.28^{a}	0.20	0.0099

$^{a-e}$Means with different superscript letters in a row differ significantly.
SEM: standard error of the mean; SL: significant level; DM: dry matter; TDM: total dry matter; CM: concentrate mix;
MLM: mulberry leaf meal; OM: organic matter; CP: crude protein; CF: crude fiber; EE: Crude fat; NDF: neutral detergent fiber;
ADF: acid detergent fiber; ME: Metabolisable energy
T1: 300 g concentrate mix + *ad libitum* barley straw;
T2: 225 g concentrate mix + 86.55 g mulberry leaf meal+ *ad libitum* barley straw
T3: 150 g concentrate mix + 173.1 g mulberry leaf meal+ *ad libitum* barley straw;
T4: 75 g concentrate mix + 259.7 g mulberry leaf meal+ *ad libitum* barley straw;
T5: 346MLM: *ad libitum* barley straw +346.2 g mulberry leaf meal

The organic matter intake was higher (P<0.05) in the diet containing sole mulberry leaf meal than concentrate mix alone. However, there was no difference (P> 0.05) in organic matter intake among T2, T3, T4 and T5 as well as among T1, T2, T3 and T4. Organic matter intake was lowest when an all concentrate diet was fed. The supplemented feeds were iso-nitrogenous and this was confirmed by the similar total CP intake of the lambs across the different treatment diets. The NDF and ADF intake was higher (P<0.05) in the diets containing sole mulberry leaf meal (T5) than T1, T2 and T3. In general, except CP intake that resulted less difference across the different treatments, other nutrients intake showed higher (P<0.05) in sole mulberry leaf meal than that of the whole concentrate mix.

Apparent dry matter and nutrient digestibility

Apparent DM, OM, CP, NDF and ADF digestibility were (P<0.05) affected by treatment effects (Table 3). The apparent DM and OM digestibility was higher (P<0.05) in sole mulberry than that of full concentrate diet. Except T1 and T5, the other treatment diets showed no (P>0.05) difference in apparent DM and OM digestibility. A higher (p<0.05) apparent NDF digestibility was obtained when larger proportion of concentrate mixture was substituted by mulberry leaf meal than the other treatment diets.

Live weight change and feed conversion efficiency

The final body weights of lambs supplemented with T3 and T4 concentrate mix as well as T5 had (p<0.05) higher than those of sheep supplemented with T1 and T2 (Table 4). However, sheep supplemented with T3 and T4 concentrate mix as well as T5 showed no difference in body weight change but T3 had significantly (p<0.05) higher body weight change than those of lambs supplemented with T1 and T2. The lambs that were fed with T3 showed higher (P<0.05) average daily weight gain (76.9 g/day) than T1 but no difference with remaining groups. Similarly, feed conversion efficiency (89g gain per gram fed) was also significantly higher (P<0.01) for lambs that were fed T3 than those fed with T1 and T2. In general, all the experimental lambs showed good growth performances throughout the experimental period.

Table 3. Dry matter and nutrient digestibility of the different treatment diets

Digestibility	Treatments					SEM	SL
	T1	T2	T3	T4	T5		
DM	0.668^{b}	0.680^{ab}	0.677^{ab}	0.685^{ab}	0.695^{a}	0.007	0.0117
OM	0.685^{b}	0.696^{ab}	0.694^{ab}	0.703^{ab}	0.711^{a}	0.007	0.0121
CP	0.738^{a}	0.728^{ab}	0.721^{b}	0.721^{b}	0.725^{ab}	0.005	0.0081
NDF	0.734^{c}	0.750^{bc}	0.752^{b}	0.772^{a}	0.774^{a}	0.006	<.0001
ADF	0.798^{c}	0.813^{ab}	0.794^{c}	0.806^{bc}	0.823^{a}	0.005	<.0001

$^{a-c}$ Means in the same row with different superscript differ significantly; DM : dry matter; OM : organic matter; CP: crude protein;
NDF: neutral detergent fiber; ADF: acid detergent fiber;
T1: barley straw *ad libitum* + 300g CM;
T2: barley straw *ad libitum* + 225g CM + 86.55g MLM;
T3: barley straw *ad libitum* + 150g CM + 173.1g MLM;
T4: barley straw *ad libitum* + 75g CM + 259.7g MLM;
T5: barley straw *ad libitum* +346.2g MLM;
SEM: standard error of mean and SL: level of significance

Table 4. Growth performance parameters of lambs fed on partial or full substituted concentrate mix by mulberry leaf meal

Growth performance parameters	Treatments					SEM	SL
	T1	T2	T3	T4	T5		
Initial body weight (kg)	17.5	17.8	17.8	17.8	18.0	0.59	0.940
Final body weight (kg)	23.2^{b}	23.6^{b}	24.7^{a}	24.6^{a}	24.5^{a}	0.42	0.005
Body weight change (kg)	5.7^{b}	5.8^{b}	6.9^{a}	6.8^{ab}	6.5^{ab}	0.39	0.012
Average daily weight gain (g/day)	63.0^{b}	64.8^{ab}	76.9^{a}	75.0^{ab}	72.2^{ab}	4.28	0.012
Feed conversion efficiency (g gain/g fed)	0.075^{b}	0.075^{b}	0.089^{a}	0.085^{ab}	0.079^{ab}	0.005	0.042

$^{a-b}$Means in the same row with different superscript differ significantly;
T1: barley straw *ad libitum* + 300g CM;
T2: barley straw *ad libitum* + 225g CM + 86.55g MLM;
T3: barley straw *ad libitum* + 150g CM + 173.1g MLM;
T4: barley straw *ad libitum* + 75g CM + 259.7g MLM;
T5: barley straw *ad libitum* +346.2g MLM;
SEM: standard error of mean and SL: level of significance

Effect on main carcass traits

Carcass characteristics of Tigray highland lambs fed full or partially substituted concentrate mix by mulberry leaf meal were assessed for slaughter weight, empty body weight, hot carcass weight, dressing percentage, rib-eye muscle area and edible and non-edible offal components. The offal components were categorized in to edible (head with tongue, heart, kidney, testis, liver with bile, empty gut, tail and fat from kidney, omentum and heart) and non-edible offal components (skin and legs, penis, lung with trachea, esophagus, spleen, bladder and gut contents) based on the eating habit of the people living around the area where the experiment was conducted.

The slaughter weight, empty body weight and rib eye muscle area indicated higher (P<0.05) in the sole mulberry leaf meal supplemented groups than those supplemented with concentrate mix alone. Moreover, hot carcass weight indicated significantly (P<0.05) higher in T3 than T1 and T2. Nevertheless there was less difference among T3, T4 and T5 for hot carcass weight and rib eye muscle area. T3 had higher (P<0.05) dressing percentage on slaughter weight base as well as empty body weight base than T1 and T2. However, it had less difference with T4 and T5.

Discussions

Feed intake

The higher total dry matter intake of sole mulberry leaf meal is in agreement with Van Soest (1994) who noted that improvement in dietary protein supplementation is due to an increase in nitrogen supply to the rumen microorganisms. This leads to an increase

Table 5. Carcass characteristics of Tigray highland lambs supplemented with graded levels of mulberry leaf meal and concentrate mix

Parameters	Treatments					SEM	SL
	T1	T2	T3	T4	T5		
Slaughter weight, kg	23.0^{b}	23.5^{b}	24.5^{a}	24.4^{a}	24.3^{a}	0.39	<.0001
Empty body weight, kg	19.3^{b}	19.8^{b}	20.7^{a}	20.5^{a}	20.5^{a}	0.36	<.0001
Hot carcass weight, kg	10.6^{b}	10.9^{b}	11.8^{a}	11.5^{ab}	11.5^{ab}	0.32	0.0003
Dressing percentage on:							
Slaughter weight base	46.1^{b}	46.4^{b}	48.0^{a}	47.1^{ab}	47.4^{ab}	0.65	0.0087
Empty body weight base	54.8^{b}	55.1^{b}	56.8^{a}	56.1^{ab}	56.4^{ab}	0.65	0.0047
Rib eye muscle area, cm^{2}	9.0^{b}	9.3^{b}	9.9^{a}	9.8^{a}	9.6^{a}	0.18	<.0001

^{a,b} Means with different superscript letters in a row differ significantly. SEM: standard error of the mean; SL: significant level;
CM: concentrate mix; MLM: mulberry leaf meal;
T1: 300 g concentrate mix + *ad libitum* barley straw;
T2: 225 g concentrate mix + 86.55 g mulberry leaf meal+ *ad libitum* barley straw
T3: 150 g concentrate mix + 173.1 g mulberry leaf meal+ *ad libitum* barley straw;
T4: 75 g concentrate mix + 259.7 g mulberry leaf meal+ *ad libitum* barley straw;
T5: 346MLM: *ad libitum* barley straw +346.2 g mulberry leaf meal;

in microbial population and efficiency, thereby enhancing the rate of breakdown of the digesta which eventually leads to increased feed intake. In line with the current study, Atiso et al. (2012) reported that increasing total DM intake with substitution of 50% of mulberry leaves for concentrate mix compared to sole concentrate mixture supplementation in dairy cows. Nevertheless, Contrary to the present study, (Alpízar-Naranjo et al. 2017)reported that the diet supplemented by sole mulberry foliage and that supplemented by whole commercial concentrate showed, respectively, the lower and higher values of total feed intake throughout the whole experimental period.

The increased NDF and ADF intake as the proportion of mulberry leaf meal increases obtained in the current study suggests the relatively increased barley straw intake observed and relatively higher fiber fraction in mulberry than that of the concentrate diet. However, the sole mulberry leaf meal supplemented sheep gained significantly (P<0.05) higher metabolizable energy than those with whole concentrate diet groups, reflecting higher digestible organic matter consumed by the lambs supplemented with sole mulberry leaf meal. According to Nguyen et al. (2005) and Doran et al. (2007), high nutritive value of mulberry forage have been recognized, and such attributes are sometimes comparable to conventional protein sources used in livestock feeding systems like soybean or alfalfa.

The high nutritive value of mulberry forage and the potential of this excellent alternative protein source forage for animal feeding in the tropics were largely discussed by González-García & Martín (2016). Mulberry forage banks respond to the objectives of looking for local animal production systems with self-sufficiency in forage production that align with whole-farm systems and address natural resource management issues such as organic matter recycling and other life cycle processes (González-García & Martín 2016). Moreover, positive animal responses have been obtained across different animal physiological or productive stages, both in meat (beef cattle, sheep and goats) and dairy (cattle, goats) production purposes (González-García & Martín 2016).

Dry matter and nutrient digestibility

The significantly (P<0.05) higher total dry matter intake observed when sole mulberry leaf meal was supplemented might be associated with improved rumen fermentation and rate of digestion without affecting cellulolytic rumen micro-organisms. The increase in intake of feed is in concordance with Van Soest (1994) who noted that improvement in dietary protein supplementation is due to an increase in nitrogen supply to the rumen microorganisms. This leads to an increase in microbial population and efficiency, thereby enhancing the rate of breakdown of the digests which eventually leads to feed intake. Hence, the sole mulberry inclusion improved dry matter intake as compared to the whole concentrates mixture suggesting the potential of mulberry leaf meal in improving intake. The less significance (P>0.05) difference in apparent digestibility of CP between most of the treatments confirmed the comparable quality of mulberry leaf meal with that of concentrate mixture.

The organic matter digestibility coefficient obtained in the current study conforms to that of Yulistiani et al.

(2015) and Desta et al. (2017) who have been noted that 0.70 and 0.71 organic matter digestibility coefficients of mature indigenous Malin rams supplemented at 1.2% of body weight with mixture of mulberry foliage (50%) and urea-rice bran (50%) and yearling intact male Abergelle sheep respectively. The CP digestibility coefficient obtained in the present study was within the range of values (68.18-76.5%) for dried mulberry leaves included at different levels in concentrate reported in different literatures (Atiso et al. 2012; T. Desta et al. 2017).

Comparable values to dry matter digestibility coefficient in the present study have been reported for dried mulberry leaves partially substituted lucerne hay and concentrates in Karagouniko sheep breed by Kandylis et al. (2009). Apparent NDF digestibility appears to be positively ($P<0.05$) affected by the inclusion of higher proportion mulberry leave meal. This result conforms to Kandylis et al. (2009) who noted due to its high digestibility and excellent level of crude protein, mulberry foliage can be a comparable source to commercial concentrates for ruminant feeding and production.

Body weight gain

The improved final weight, body weight change and average daily gain when concentrate was partially or fully substituted by mulberry leaf meal were attributed to the differences in digestibility and intake of DM as well as OM observed. Rubanza et al. (2007) also indicated a better growth performance when meals of forage trees like *A. nilotica*, *A. polyacantha* and *L. leucocephala* were supplemented in combination with other concentrate for goats that is in support of the current finding. Similarly, Alpízar-Narajo et al. (2017) reported that the differences found in dry matter intake could be the indirect resultant of the differences in the energy feed sources offered to each group, i.e. different energy pools coming from concentrate and/or mulberry. In line with the present finding, diet supplementation with mulberry leaves has been reported to lead in increased body weight gains in growing lambs (Benavides et al. 2000) and growing goats (Gonzalez & Milera 2000). Moreover, Ba et al. (2005) also found that milk production increased with the levels of mulberry offered to goats. Benavides et al. (2000) also observed no difference in milk yield among groups of grazing dairy cattle supplemented with either concentrate or mulberry leaves.

Replacing the mulberry for soybean meal in diets for dairy cows did not affect milk yield or quality (Cuong et al. 2007). Positive results including different levels of mulberry have been reported by others in the feeding of sheep (Pacheco et al. 2002).

Carcass characteristics

The higher slaughter weight and empty weight resulted in sole mulberry leaf meal as compared to the whole concentrate mix as well as the less differed dressing percentage on empty body weight base and dressed hot carcass weight across the different treatments might justify the potential of mulberry leaf meal to replace partially or fully to the commercial concentrate mixture. Dressing percentage based on empty body weight (54.4-56.8 %) obtained in this study was comparable to 53-56.3% (Gemechu & Mekasha 2012), 53-57% (Legesse 2008) and 55.7-56.4% (Abreha 2011). However, Gebresilassie (2011) and Gebresilassie (2007) reported lower results of dressing percentage based on empty body 48.6-50% and 47-53% respectively, than the current study.

The dressing percentage based on slaughter weight base in the current study ranged between 46.1-48.0%, which seemed in agreement with the 47.3-48.6% for Tigray highland sheep (Degu et al. 2009). Nevertheless, lower results on dressing percentages of 32-38%, 38-39.6%, 39.5-43.4% and 36-38.4% on slaughter weight basis than the current study were reported by Gebresilassie (2011) and Mezgebo & Urge (2011) for local sheep, Gebresilassie (2007) for Arado sheep and Moges (2005) for Wogera sheep, respectively. Generally, the variations in carcass traits in this study and other results of previous studies might be due to variations in age and breed of sheep, and quantity and quality of basal and supplement feeds used during the experiment. In agreement with this, (McDonald et al. 2010) noted that, nutrition, age, sex, genetics, season and other related factors affect the growth and carcass traits of animals.

Rib eye muscle area is an indicator of muscling and amount of lean meat in the carcass. The rib eye muscle area in the current study was in the range of 9.0-9.9 cm^2 that is comparable with 8.6-9.5, 6.3-9.2 and 8.2-10.4 cm^2 reported by Gemechu & Mekasha (2012), Degu et al. (2009) and (Legesse 2008) for supplemented groups of Black Head Ogaden sheep, Tigray Highland sheep and Arsi-Bale sheep, respectively. Lower values of rib eye muscle area than the present study were reported by Desta et al. (2017) (8.43-8.98 cm2), Fesaha & Urge (2014) (6.7-7.3 cm2), Gebresilassie (2011) (4.5-6.5), Abreha (2011) (5.7-6.4 cm2) and Yirga (2008) (7-8.4 cm2) for yearling intact male Abergelle sheep, Black Head Ogaden sheep, local sheep, Tigray Highland sheep and Hararghe Highland sheep, respectively. However, rib eye muscle area in the current study was lower than the values of 13-19.5 and 11.5-12.75cm2 reported by Moges (2005) and Gebresilassie (2007) for supplemented groups of Wogera sheep and Arado sheep, respectively. The differences in rib eye muscle

area reported by various authors might be due to variations in the amount and quality of supplements and variations in sheep breeds used for the experiment. Rib eye area is affected by the weight and muscularity of the live animal (O'Rourke et al. 2004) and it is increased with carcass weight (Park et al. 2002) and this report conforms to the result of the current finding.

CONCLUSION

The sole mulberry leaf meal supplementation improved dry matter intake as compared to the whole concentrates mixture suggesting the potential of mulberry leaves in improving intake. Apparent NDF digestibility appears to be positively affected by the inclusion of higher proportion of mulberry leaf meal. The study revealed that supplementing mulberry leaf meal alone to lambs fed based on barley straw could replace concentrate and result in reasonably good performance. The result of the present study also indicated that substitution of mulberry leaf meal for concentrate mixture can be used effectively without affecting the performance of lambs that can be achieved by sole concentrate mixture supplementation.

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Effect of Supplementation of BS4-Enzyme Levels in Rice-Bran Based Rations on Performance of Growing PMp Broiler Duck

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ABSTRAK

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Suplementasi enzim dalam ransum antara lain bertujuan untuk meningkatkan kecernaan gizi melalui degradasi anti-nutrisi dan serat kasar yang terdapat dalam dedak padi. Tujuan penelitian ini adalah untuk menguji efektifitas suplementasi enzim BS4 dalam ransum itik pedaging PMp berbasis dedak padi. Dua ratus dua puluh empat ekor itik PMp umur sehari dipelihara selama empat minggu dan dialokasikan secara acak ke dalam 8 perlakuan dengan 4 ulangan dan setiap ulangan terdiri dari 7 ekor itik. Ransum perlakuan dengan notasi T1 - T4 adalah ransum dengan kadar dedak padi 30%, dengan kadar enzim BS4 masing-masing 0, 50, 100, 150 Unit/kg dedak padi. Ransum perlakuan T5 - T8 adalah ransum dengan kadar 60% dedak padi,dengan kadar enzim sama dengan pada ransum perlakuan T1 – T4. Peubah yang diamati meliputi: konsumsi pakan, pertambahan bobot badan (PBB) dan FCR. Hasil penelitian menunjukkan bahwa suplementasi enzim BS4 dalam ransum berbasis dedak (30 dan 60%) berpengaruh nyata (P<0,05) terhadap konsumsi pakan dan FCR, akan tetapi tidak untuk PBB (P>0,05). Ransum perlakuan yang paling efesien mendukung pemeliharaan itik adalah ransum perlakuan T4, yang menunjukkan rataan konsumsi pakan terendah (998g/ekor) dan FCR terendah (2,64). Disimpulkan bahwa suplementasi enzim BS4 sebesar 150 Unit/kg dalam ransum berbasis dedak padi 30% merupakan kadar yang paling baik untuk mendukung pertumbuhan terbaik itik pedaging PMp selama pemeliharaan 4 minggu pertama.

Kata Kunci: Enzim BS4, Dedak Padi, Itik Pedaging PMp

ABSTRACT

Purba M, Sinurat AP. 2018. Effect of supplementation of BS4-enzyme levels in rice-bran based rations on performance of growing PMp broiler duck. JITV 23(1): 38-44. DOI: http://dx.doi.org/10.14334/jitv.v23i1.1669

The purpose of enzymes supplementation in feeds is to improve nutrient digestibility through degradation of anti-nutrition and crude fiber, which are commonly found in rice bran. The aim of the study was to see performance response of PMp broiler ducks to the supplementation of BS4-enzyme levels in rice-bran based rations. Two hundred and twenty four day-old ducks were allocated to 8 dietary treatments with 4 replicates, consisted of 7 ducks in each replicate. The composition of the feed treatments arranged as follows: T1 to T4 were rations with 30% of rice bran content with enzyme levels of 0, 50, 100, and 150 Unit/kg rice bran respectively. T5 to T8 were rations with 60% of rice bran content with the same enzyme levels as for T1 to T4 treatments. The ducklings were subjected to the treatments for the first four weeks. The variables observed were feed intake, weight gain and FCR. The results showed that the supplementation of BS4-enzymes on rice-bran based rations significantly affected (P<0.05) feed intake and FCR, but not for weight gain (P>0.05). The most effective rations for feed consumption and FCR of PMp broiler duck were obtained on T4 treatment resulting in the highest body weight gain of 998 g/bird and lowest FCR of 2,64. It was concluded that the supplementation of 150 Unit/kg of BS4 enzyme in 30% rice-bran diet was the best combination level to be implemented in feeding PMP broiler ducks for the first four weeks period.

Key Words: BS4 Enzyme, Rice Bran, PMp Broiler Duck

INTRODUCTION

Duck meat is one of the interesting poultry products. Demand for duck meat lately seems to increase. During the last five years duck meat production in Indonesia has also increased although the percentage of the increase was still low ranging from 3-7 per cent (DGLAH 2016). Since the production of local duck meat has not been sufficient for the domestic market, the government of the Republic of Indonesia has made policy concerning importation of duck meat. The type of imported duck meat is frozen Pekin duck meat as it has high quality meat according to market in Indonesia

One of the efforts to meet the needs of duck meat, Indonesian Research Institute for Animal Production (IRIAP) has invented a new breed of broiler ducks, namely PMp ducks, obtained from crossing of male Pekin duck with female white Mojosari duck. The PMp has advantages of rapid growth, body live weight above 2 kg/bird at the age 10 weeks, and clean white carcass

color. In addition, The PMp is actually developed as broiler type, however, it has also good average egg production, reaching of 65-70% per year. The drawback of the Indonesian meat type ducks was having high FCR, ranging from 3.97 to 4.70 (Ketaren et al. 2011). Therefore it is necessary to carry out research, considering nutrition and feed technology by the use of enzymes as a supplement in duck feed.

Enzymes are organic molecules (proteins) can serve as catalysts or accelerate certain chemical reactions. Some of the benefits of using enzymes in livestock are improving growth and feed conversion ration, controlling health by preventing digestive disorders, especially in young animals (Havenaar et al. 1992). It was further reported that the use of enzymes in livestock could facilitate the digestion process of anti-nutritional factors such as trypsin, phitic acid and glucosinolate (Havenaar et al. 1992). Enzymes could improve nutrient digestibility by breaking up complex molecular structures into simpler molecular structures (e.g, from polysaccharides to monosaccharides or from proteins to amino acids (Borin et al. 2002, Hernandez et al. 2004; Kong & Adeola 2010; Rutherfurd et al. 2007).

The use of enzymes is one of the right actions because it has no negative side effects when administered in the proper dosage. Enzymes are non-toxic, natural and immediately inactive when the reaction has reached the desired point (Patterson & Burkholder 2003; Cavazzoni et al. 1998; Yeo & Kim 1997). The addition of enzymes in laying chicken and broiler chicken diets has been reported to have positive respons for poultry. Xuan et al. (2001) reported that administering 0.10-0.30% of commercial enzymes in rations, improved phosphorus digestibility, growth, and efficiency of ration use in broiler chickens. The commercial enzyme is a combination of several enzymes such as alpha-amylase, xylanase, beta-gluconase, protease, lipase, and phytase (Xuan et al. 2001). Other researchers have also reported that supplementation of phytase enzymes to the rations improved the digestibility of crude protein, P, Zn, Mg, and Cu, and increased the retention nitrogen of Ca, P, Mg, and Zn minerals (Lim et al. 2001). Simbaya et al. (1996) reported that supplementation of enzyme (phytase, carbohydrase and protease) in rations increased weight gain and the efficiency of ration on broiler chickens. It was further reported that the nutrients digestibility increased with the supplementation of the three enzymes. The addition of enzyme (protease, cellulase, and hemicellulase) was found to increase the growth and efficiency of ration (Selle et al. 2003).

Pasaribu et al. (2009) reported that the addition of BS4 enzyme could increase metabolic energy and solid-phase solidity (SHP), whereas with single commercial enzymes only increased metabolic energy. The optimum dose of BS4 enzyme addition was 13.3 ml. Enhanced SHP, when used in laying hens, replacing the use of maize up to 25%, without causing a decrease in egg production and egg quality (Sinurat et al. 2007; 2008).

Information on the supplementation of enzymes on feed of laying duck has been reported, but for the broiler type of duck was still few, especially in local ducks. Candrawati et al. (2006) reported that phylazim enzyme supplementation of 0.20% in rations containing 30% rice bran was not significantly different from rationing using 15% rice bran and without enzyme. Chandrawati et al. (2006) reported that the use of rice bran up to 30% with the addition of enzymes in broiler aging 2-6 weeks was feasible.

Rice bran generally contains high fiber that is not easy to digest. It contains high fiber, protein and energy, stored in the cell wall in the form of cellulose and hemi cellulose, which is difficult to digest by poultry. The use of rice bran with a high proportion by the farmers in the field has been going on even though the duck production yield is very low. Rice bran besides containing high fiber it is also containing anti-nutrients such as phytic acid. The anti-nutrients can bind proteins, starches and minerals so that their presence in the diet can inhibit the digestibility of proteins, starches and minerals. Gallinger et al. (2004) suggest that the use of rice bran in poultry feed should not exceed 20% because it contained high crude fiber. These conditions can inhibit the digestion process, feed absorption so that it can disrupt the growth of ducks. All the obstacles are expected to be overcome by the addition of enzymes in the feed. The purpose of this paper was to determine the effect of supplementation of various levels of BS4-enzyme based on high level (30 and 60%) of rice bran on diet to the performance of PMp broiler duck.

MATERIALS AND METHODS

The material used was PMp broiler ducks obtained from crossing of male Pekin duck through artificial insemination (AI) with female Mojosari duck (one of new IRIAP's duck strain). The whole process of keeping the parent ducks was conducted according to standard operational research of IRIAP. PMp ducklings were then allocated randomly in 32 wire cages, equipped with feed and drinking water. The ducklings were reared from the age of 0 to 4 weeks. The data were then analysed using completely randomized design (CRD) 2 x 4 pattern. The first factor was two levels of rice bran of 30 and 60%, the second factor were four BS4-enzyme levels of 0, 50, 100, and 150 Unit/kg rice bran. So, there were eight treatments, each treatment had four replicates, each replicate consisted of seven ducks. The enzyme used were BS4-enzyme, which was produced in IRIAP by utilizing coconut cake, mineral

mixture and mocrobe of *Eupenicillium javanicum* (Pasaribu et al. 2009; Sinurat et al. 2016). The dietary treatments followed:

T1, diet + 30% rice bran + 0 Unit BS4-enzyme/kg
T2, diet + 30% rice bran + 50 Unit BS4-enzyme/kg
T3, diet + 30% rice bran + 100 Unit BS4-enzyme/kg
T4, diet + 30% rice bran + 150 Unit BS4-enzyme/kg
T5, diet + 60% rice bran + 0 Unit BS4-enzyme/kg
T6, diet + 60% rice bran + 50 Unit BS4-enzyme/kg
T7, diet + 60% rice bran + 100 Unit BS-enzyme/kg
T8, diet + 60% rice bran + 150 Unit BS4-enzyme/kg

The nutrient content of rations was formulated based on nutritional requirement for duck following the recommendations of NRC (1994), Chen (1996), and Ketaren et al. (2010). The treatment rations were prepared iso protein and iso energy. The rations, which were provided at the age of 1 to 7 days was a commercial starter ration. Provision of the treatment ration with enzyme supplementation and high bran content was carried out since duck entered the age of one week. Materials and the composition of the treatment are presented in Table 1. Feed was given twice a day in the morning and afternoon, while drinking water was provided *ad libitum*. The variables measured were feed intake, body weight gain, and feed conversion ratio (FCR). Ducks were weighed once a week to obtain body weight data as well as consumption and feed efficiency. Data were analyzed by using the procedure of General Linear Model (GLM) applying Statistical Analysis System (SAS, ver. 6.12, 1997).

Table 1. The composition and nutrient content of the dietary treatments

Ingredients	Feed treatments							
	T1	T2	T3	T4	T5	T6	T7	T8
Rice bran, (%)	30.00	30.00	30.00	30.00	60.00	60.00	60.00	60.00
Corn, (%)	34.60	34.60	34.60	34.60	6.21	6.21	6.21	6.21
Soybean meal, (%)	22.06	22.06	22.06	22.06	16.53	16.53	16.53	16.53
Fish meal, (%)	5.88	5.88	5.88	5.88	8.53	8.53	8.53	8.53
Methionin, (%)	0.15	0.15	0.15	0.15	0.12	0.12	0.12	0.12
Lysine, (%)	0.42	0.42	0.42	0.42	0.45	0.45	0.45	0.45
Premix, (%)	0.40	0.40	0.40	0.40	0.05	0.05	0.05	0.05
Crude palm oil, (%)	5.50	5.50	5.50	5.50	7.95	7.95	7.95	7.95
Dicalcium posphate, (%)	0.55	0.55	0.55	0.55	0.10	0.10	0.10	0.10
Lime stone, (%)	0.15	0.15	0.15	0.15	0.03	0.03	0.03	0.03
Salt (%)	0.29	0.29	0.29	0.29	0.03	0.03	0.03	0.03
Total	100	100	100	100	100	100	100	100
BS4 Enzyme (Unit/kg)	**0**	**50**	**100**	**150**	**0**	**50**	**100**	**150**
Nutrient content (% dry matter)*								
Protein, (%)	19.29	19.29	19.29	19.29	19.02	19.02	19.02	19.02
Energy, (kkalME/kg)	2933	2933	2933	2933	2896	2896	2896	2896
Crude fiber, (%)	5.21	5.21	5.21	5.21	8.64	8.64	8.64	8.64
Methionine, (%)	0.33	0.33	0.33	0.33	0.31	0.31	0.31	0.31
Lysine, (%)	1.05	1.05	1.05	1.05	1.05	1.05	1.05	1.05
Calcium, (%)	0.87	0.87	0.87	0.87	0.97	0.97	0.97	0.97
Phosfor, (%)	0.71	0.71	0.71	0.71	0.90	0.90	0.90	0.90

*) The nutrient content based on calculation; ME (Metabolisable energy)

RESULTS AND DISCUSSION

Feed consumption

The average feed consumption of PMp broiler duck fed with rations based on two levels of rice bran (30 and 60%) and supplemented with BS4 enzyme is presented in Table 2. There was no significant (P>0.05) interaction between supplementation of BS4-enzyme and rice bran. Feed consumption was affected (P<0.05) by supplementation of BS4-enzyme but not by the rice bran levels (P> 0.05). Supplementation of BS4-enzyme with doses of 150 Unit/kg and 30% of r ice bran in ration resulted in the lowest feed intake compared to other treatments. The average feed consumption of ducklings in the T4 treatment was 998 g/4 week/duckling. The result of this study was in line with the report of Simbaya et al. (1996), Xuan et al. (2001) and Shelle et al. (2003).

The average feed consumption resulted appeared to be decreasing along with the increasing BS4-enzyme level in all the treatment rations. The average feed consumption with T4 treatment ration (150 Unit BS4-enzyme/kg in 30% rice bran ration) decreased by 14.7% of control rations, whereas in T2 and T3 treatment decreased by 0.68 and 0.85%. The decrease in consumption of ration contained 60% bran, was almost equal to the decrease consumption in T2 and T3 treatment (30% of rice bran). The results illustrated that the higher dose of BS4-enzyme supplemented to the ration, the more ability the ducklings to change the complex molecular structure into a simple form and simplify the process of feed digestion.

Havenaar et al. (1992) stated that the use of enzymes in animals could eliminate various anti-nutritional factors present in the diet such as trypsin, phitic acid and glucosinolate and facilitate the digestion process in poultry. Digestion process was increased by the availability of enzyme causing the increase in nutrients availability and absorption of the nutrient consumed by PMp ducks. Selle et al. (2003) stated that the addition of enzymes (protease, cellulase, and hemicellulase) in animal feeds, improved feed efficiency by increasing growth in broiler chickens. Table 2 also shows that the average feed consumption with addition of BS4-enzyme (150 Unit/kg) was significantly improved (P<0.05) duckling performance compared the other treatments. The study indicated that supplementation of BS4-enzyme by a dose of 150 Unit/kg in 30% rice bran the diet, was the best combination levels to decrese broiler duck feed consumption during the first four weeks of age.

Body weight gain

The average weight gain of PMp broiler ducks fed rations contained two levels of rice bran (30 or 60%) supplemented with BS4-enzyme up to 4 weeks of age is presnted in Table 3. BS4-enzyme supplementation to rations contained two levels of rice bran up to 4 weeks of age did not significantly increase (P>0.05) body live weight gain of the PMp broiler duck.

Tabel 2. The average feed consumption of PMp boiler ducks fed rations contained 30 or 60% rice bran supplementation with various levels of BS4-enzymes up to 4 weeks of age

Rice bran levels	Enzyme levels (Unit/kg rice bran)				Average
	0	50	100	150	
30%	1170±53.88*[a]	1162±68.00[a]	1160±64.00[a]	998±22.60[b]	1123
60%	1164±23.50[a]	1161±23.36[a]	1160±24.00[a]	1160±24.00[a]	1161
Average	1167[a]	1161[a]	1160[a]	1079[b]	

Values in the same column with different superscript are significantly difference (P<0.05)
*) Standard error

Table 3. The average body live weight gain of PMp boiler ducks fed rations contained 30 or 60% rice bran supplementation with various levels of BS4-enzymes up to 4 weeks of age

Rice bran level	Enzymes level (Unit/kg rice bran)				Average
	0	50	100	150	
30%	733.00±24.81*	712.75±27.99	714±21.27	785.50±14.62	736.25
60%	724.00±23.01	727.25±43.77	715±32.68	695.25±33.21	715.50
Average	728.50	720.00	714.68	740.38	

*) Standard error

Sinurat et al. (2007) reported that the addition of BS4-enzyme in laying hens did not effect (P>0.05), body live weight gain, although egg production, egg weight and feed efficiency increased (P<0.05). Sinurat et al. (2016) concluded that supplementation of BS4-enzyme in laying hens diet did not influence body weight, feed intake, mortalities, egg weight and egg shell thickness. However it was further reported that supplementation BS4-enzyme in the diet contained palm kernell cake (PKC), improved the egg yolk color score but no effect on maize or rice bran diets (Sinurat et al. 2016). Tirajoh et al. (2010) has also reported that the addition of phytase enzyme to the ration containing 30% rice bran up to 6 weeks of broiler chicken did not significantly affect live body weight gain (P> 0.05).

Kochcer (2003) and Olukosi et al. (2007) had also reported that the addition of xylanase, amylase and protease enzymes in the feed did not significantly (P>0.05) affect body live weight gain of broiler up to 21 days of age. Other research results as reported by West et al. (2007) showed that the addition of xylanase and ß-glucanase enzyme in corn and soybean meal did not significantly influence to the body weight of broiler chickens.

The average body live weight gain of PMp ducks ranged from 695.25 to 785.50 g/bird up to 4 weeks of age. Sinurat et al. (2007) reported that the addition of BS4 enzyme in laying hens did not effect (P>0.05), but increased egg production, egg weight and feed efficiency. Sinurat et al. (2016) concluded that supplementation of BS4-enzyme in the laying hens diet did not influence the body live weight change, feed intake, mortalities, egg weight and egg shell thickness. However it was further reported that supplementation BS4 enzyme into the diet improved the egg yolk color score of chicken fed the PKC diet but not affected when fed maize or rice bran diet (Sinurat et al. 2016). Tirajoh et al. (2010) has also reported that the addition of phytase enzyme to the ration containing 30% rice bran to 6 weeks old of broiler chicken did not significantly affect body weight (P>0.05).

The results showed that the highest body weight gain was found in T4 treatment (785.50 g/bird) increased by 7.16% compared to the control ration, while the lowest was found in the T8 treatment of T8 (695.25 g/bird), which was decreasing by 3.97% of control rations. In Table 3 appears that supplementation of 150 Unit BS4-enzyme/kg in ration containing 30% rice rice bran, increased body live weight gain of the duckling. In contrast to the results achieved in rations containing 60% of rice bran, although the BS4 enzyme level was raised up to 150 Unit/kg the body live weight gain of the duck was even decreased. Table 3 shows that except for the T6 treatment (50 Unit BS4 enzymes/kg), the increase in body weight of PMp duck under T7 treatment (100 Unit/kg) and T8 (150 Unit/kg) was lower compared than the control ration (T5). The study was in line with Gallinger et al. (2004) who stated that the body weight gain of broiler chicken decreased along with the increase of rice bran rate by 30 and 40%, causing the decrease about 3.6 and 8% respectively compared to control ration. When viewed from aspect of the effectiveness, the addition of BS4-enzyme at a dose of 150 Unit /kg in 30% rice bran diet was considered adequate to support the increased body weight gain of PMp broiler duck up to 4 weeks of age.

Feed Conversion Ratio (FCR)

The average FCR of PMp broiler duck with under rice bran based rations (30% or 60%) supplemented with various levels of BS4-enzyme are described in Table 4. Based on the results of the analysis of the varians, the influence of the interaction between the various levels of BS4-enzyme with both rice bran levels in feed treatment was not significant (P>0.05). The BS4-enzyme supplementation in to 30% rice bran diet had significant effect (P<0.05) on FCR, but did not in 60% rice bran diet. BS4-enzyme supplementation with a dose of 150 Unit/kg in 30% rice bran rations, resulted in the lowest FCR compared to other treatments. The average FCR produced on the T4 treatment was 2.64. Sinurat et al. (2007) reported that the FCR of laying chicken was decreasing along with the addition of BS4-enzyme in the ration. Other research also have been reported by Tirajoh et al. (2010), who stated that the efficiency of broiler chicken up to the age of 6 weeks increased by the addition of phytase enzyme into the ration.

Table 4. The average feed conversion ratio (FCR) of PMp broiler ducks fed rations contained 30 or 60% rice bran supplementation with various levels of BS4-enzymes up to 4 weeks of age

Rice bran levels	Enzyme levels (Unit/kg rice bran)				Average
	0	50	100	150	
30 %	3.17±0.07*[a]	3.23±0.14[a]	3.19±0.08[a]	2.64±0.13[b]	3.05
60 %	3.08±0.09[a]	3.07±0.18[a]	3.16±0.28[a]	3.22±0.35[a]	3.13
Average	3.13	3.15	3.17	2.93	

Values in the same column with the different superscript are significantly difference (P<0.05)
*) Standard error

The FCR value in duck farming business is very important considering that ducks consume a lot of feed. Ducks are very rare to stop consuming feeds as long as necessary nutritional requirements are not yet fulfilled. Therefore it is important to know the quality and nutritional content of any feedstuffs that will be given to ducks. Cowieson (2010) recommended that one of the strategies to improve nutritional content of feed include energy content, was enzymes supplementation with enzymes. Addition of enzymes was lowering feed consumption as well as feed costs. Similar to Cowieson (2010), other researchers also reported that the addition of enzymes into the diet increased the nutritional content of proteins and amino acids and its digestibility (Zanell et al. 1999; D'Alfonso 2005; Cowieson & Ravindran 2008). The low FCR resulted in T4 treatments, illustrated the role of BS-4 enzyme in digestion of crude fiber and anti-nutrient substances. It also helped nutrients absorption more effective than other treatments. The supplementaton of BS4-enzyme at a dose of 150 Unit/kg in diet containing of 30% rice bran was most effective to produce low FCR in PMp broiler duck up to 4 weeks of age. The study was in line to the reports of Simbaya et al. (1996), Xuan et al. (2001) and Shelle et al. (2003) who stated that the efficiency of feed consumption has increased along with the enzyme supplementation in broiler rations. The results showed that the addition of BS4-enzyme in ration containing rice bran by 30% could improve feed efficiency in PMp duck up to 4 weeks of age.

CONCLUSION

The supplementation of BS4-enzyme in rice bran based diet was effectively to enhance performance of PMp broiler duck up to weeks of age. Supplementation of 150 Unit BS4-enzymes/kg rice bran in ration containing 30% of rice bran, effectively increased feed efficiency of PMp broiler ducks up to 4 weeks of age.

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Epidemiology of Traumatic Myiasis due to *Chrysomya bezziana* in Indonesia

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ABSTRAK

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Epidemiologi myiasis traumatika di Indonesia dipelajari melalui larva lalat yang dikoleksi dari ternak di berbagai daerah dengan melakukan surveilan pasif yang melibatkan Pos Kesehatan Hewan (Poskeswan). Data bulanan kasus myiasis di Kediri – Jawa Timur pada tahun 2006-2009 juga dianalisis untuk memperoleh gambaran kejadian musiman myiasis di daerah tersebut. Larva yang dikoleksi dari 260 kasus myiasis di berbagai daerah dan 341 kasus di Kediri diidentifikasi. Berdasarkan hasil identifikasi, seluruh penyebab myiasis traumatika di Indonesia adalah lalat *Old World Screwworm* (OWS), *Chrysomya bezziana* (Diptera : Calliphoridae) kecuali lima kasus pada ayam yang disebabkan oleh spesies lalat *Musca sp*. Jumlah kasus myiasis traumatika setiap bulan di Kediri sangat bervariasi, namun demikian peningkatan kasus secara nyata terjadi pada bulan Januari dan Desember pada saat musim hujan. Jumlah kasus infestasi larva OWS tertinggi pada studi ini adalah sapi dan kambing. Hasil ini membuktikan bahwa ternak sapi memiliki resiko terserang myiasis traumatika lebih tinggi dibandingkan ternak yang lain. Vulva dan umbilikus (tali pusar) adalah bagian tubuh yang paling sering terserang myiasis dan memiliki korelasi positif dengan proses beranak. Analisis DNA mitokondria terhadap 176 sampel membuktikan bahwa marka ini mampu digunakan untuk mendeteksi adanya multi-infestasi, namun tidak dapat menunjukkan korelasi positif antara garis keturunan tertentu dengan inangnya. Kombinasi iklim Indonesia yang terletak di garis katulistiwa dan sistem peternakan yang masih traditional menjadi faktor utama berkembangnya lalat OWS sepanjang tahun. Apabila myiasis tidak mendapat perhatian, maka akan menjadi ancaman peternakan di Indonesia termasuk menjadi masalah terhadap kesejahteraan para peternak. Data epidemiologi pada studi ini merupakan data myiasis traumatika yang cukup lengkap dan menjadi studi yang penting dalam mendukung program manajemen hama yang terintegrasi.

Kata Kunci: Myiasis, Mitokondria, *Chrysomya bezziana*, Epidemilogi

ABSTRACT

Wardhana AH, Abadi I, Cameron MM, Ready PD, Hall MJR. 2018. Epidemiology of traumatic myiasis due to *Chrysomya bezziana* in Indonesia. JITV 23(1): 45-60. DOI: http://dx.doi.org/10.14334/jitv.v23i1.1617

Epidemiology of traumatic myiasis in Indonesia was studied by the widespread collection of fly larvae from infested livestock in passive case detection surveys involving veterinary clinics. In addition, monthly data from Kediri regency in Eastern Java were analysed from 2006-2009 to explore the seasonality of myiasis. Larvae from a total of 260 cases from the nationwide survey and 341 cases from Kediri were identified. Except for 5 cases of chicken infestation due to *Musca species* in the nationwide survey, all other cases were exclusively caused by the Old World screwworm (OWS) fly, *Chrysomya bezziana* (Diptera: Calliphoridae). The monthly numbers of cases at Kediri were very variable, with cases in all months, but there was statistical evidence for an increase in cases in January and December, during the rainy season. The greatest numbers of infestations recorded were from cattle and goats. The most frequently infested sites nationwide and in Kediri were the vulva and umbilicus, associated with calving, which is a major risk period for traumatic myiasis. Mitochondrial DNA typing of 176 specimens was useful for detecting multiple infestations, but no association was found between genetic lineage and host. The equatorial climate of Indonesia, combined with poor husbandry systems are factors that help to support OWS fly development year round. Even if not considered a disease of strategic importance, screwworm myiasis remains a threat to livestock production in Indonesia and a major welfare issue that requires constant interventions by farmers. The new and collated epidemiological data presented represent the most extensive survey of traumatic myiasis in Indonesia to date and provide a valuable baseline to support integrated pest management programmes.

Key Words: Myiasis, Mitochondria, *Chrysomya bezziana*, Epidemiology

INTRODUCTION

The larvae of the Old World screwworm (OWS) fly, *Chrysomya bezziana* (Diptera: Calliphoridae), cause traumatic myiasis and remain a major problem in sub-Saharan Africa and Asia (Hall et al. 2016). The disease is considered to be a serious animal health and welfare problem in the central livestock regions of Indonesia, particularly in the East where livestock are raised freely in the field for their entire life, a practice called the extensive husbandry system (Partoutomo 2000). The disease is found in both traditional and commercial farms and cases occur throughout the year (Wardhana et al. 2014).

Since the first report in 1926, in Minahasa, North Sulawesi (Kraneveld & Schaaf 1937), myiasis has continued to be a livestock problem in Indonesia, and since 1948 the fly causing most infestations has been identified as the OWS fly (Wardhana et al. 2014). However, this disease is not considered to be of strategic national importance and therefore livestock owners rather than veterinarians apply treatments that are highly diverse in manner and outcome. In particular, because effective insecticides, such as Dichlorfenthion (Gusanex®) favoured by veterinarians, are expensive and difficult to find in the markets, less affluent rural farmers depend instead on herbal medicines to kill the larvae in the wound, such as liquid of tobacco extract and others (Sukarsih et al. 1989; Wardhana & Diana 2014; Mustika et al. 2016; Wientarsih et al. 2017). They also traditionally used kerosene, battery carbon with motor oil, gasoline and petrol to treat myiasis (Sukarsih et al. 1989). These methods might kill the larvae, but lead to skin irritation rather than wound healing. In East Sumba, many farmers additionally used insecticides developed for control of plant pests such as Isoprocarb 50% WP (MIPCIN 50 WP®), which is potentially dangerous for livestock and can cause poisoning, even death (Wardhana 2006).

Partoutomo (2000) reported three main reasons why acute outbreaks of traumatic myiasis still occur in Indonesia. Firstly, the primary myiasis agent, the OWS fly, is distributed throughout the Indonesian archipelago; secondly, the environment and warm tropical weather with high humidity are suitable for development of *C. bezziana* almost throughout the year; and thirdly, a large supply of livestock hosts are available year-round, many of which are raised in an extensive livestock management system where they are not always inspected daily and so not treated in a timely manner if infested. Based on observations on commercial farms, it was concluded that imported livestock and those raised under an extensive grazing system are at high risk for myiasis infestation (Sigit & Partoutomo 1991; Wardhana 2006). The World Organisation for Animal Health (OIE) provides country and disease data related to animal health that is held in the World Animal Health Information Database (WAHID) interface. According to the WAHID interface, myiasis cases remain a threat in many tropical countries. For example, Malaysia provided annual reports to WAHID between 2005-2014 which indicate that myiasis due to the OWS fly persists there. However, unlike its northerly neighbour, Indonesia has not submitted information on myiasis to WAHID. Clearly, this lack of reporting gives a wrong impression of the situation, because the disease is endemic and widely distributed throughout Indonesia (Wardhana et al. 2003; Wardhana et al. 2014).

The Sterile Insect Technique (SIT) has been successfully applied to eradicate a primary myiasis agent, *Cochliomya hominivorax*, from the North American continent and more recently from Libya and from most countries of Central America (Welch & Hall 2013). One of the reasons for successful eradication of *C. hominivorax* in Libya using the SIT was due to a good understanding of interrelated components such as epidemiology and surveillance of the disease, treatment techniques, rigorous animal inspection and quarantine, and a good communication between farmers, veterinarians and other stakeholders (Lindquist et al. 1992). Robinson et al. (2009) stated that the collection of epidemiological data is the central source of management information for SIT programmes, because the data are used to determine the status and severity of the infestations and the economic magnitude of the problem. In addition, epidemiological data are correlated with the release rate of sterile insects to maximise the effect of the programme in eradicating *C. hominivorax* (Cox & Vreysen 2005). Trials in Papua New Guinea revealed that the SIT should also be effective against *C. bezziana* (Spradbery et al. 1989; Mahon 2002).

However, before the SIT is applied in any country, the essential first steps are to collect comprehensive baseline data, including the distribution of myiasis, assessment of ecological and genetic data and also the infestation dynamics (Hall et al. 2001; Vargas-Terán et al. 2005). Moreover, Cox & Vreysen (2005) proposed that mapping a pest's distribution on a regional and national scale is a fundamental phase to assess the feasibility and spatial targeting of the SIT. Modelling of the distribution of the OWS fly in Indonesia was undertaken by Wardhana et al. (2014), but this included only records that could be geo-referenced. Therefore, the present study reports all known presence records by island, province and regency. It focuses on the infestation dynamics and epidemiology of OWS myiasis in Indonesia, including the seasonal dynamics in Kediri regency and potential behavioural differences between OWS flies characterised by different

mitochondrial DNA lineages (Wardhana et al. 2012) in Banten and Kediri regencies, all on Java Island.

MATERIALS AND METHODS

Surveillance for myiasis of livestock and collection of infesting fly larvae

Three kinds of surveillance were co-ordinated by the Indonesian Research Center for Veterinary Science (IRCVS), Bogor, Java Island. 'Collaborative sampling' was performed by the IRCVS and a regional team during field trips of 1-2 weeks, after which the regional team was encouraged to carry out 'Continuing sampling' following the same protocols. 'Oriented sampling' was performed solely by a regional team, based on a protocol communicated by IRCVS. All three approaches were effectively passive case detection, because livestock owners were only asked to bring infested animals to a sampling point, not to carefully screen all body parts of each of their animals for any wounds.

'Collaborative sampling' was carried out by visiting a total of 170 farms, both traditional and commercial, during three years, 2005, 2007 and 2009 on the islands of Borneo, Sumatra, Java, Lombok, Sumbawa, Sumba and Sulawesi (Figure 1). 'Oriented sampling' was undertaken on these islands and others (Madura, Timor and New Guinea) 2002-2009 by contacts in local veterinary clinics, where the staff collected larvae from cases of myiasis without any selection based on species identification. Fly larvae were sampled from wounds and preserved according to a protocol (see below) that was sent to local veterinarians, livestock agencies and Disease Investigation Centres (DICs), and all samples were then sent to the IRCVS in Bogor.

Preservation and identification of fly larvae from myiasis wounds

Larvae were collected from wounds using forceps, killed by immersion in boiling water for *c.* 15 seconds (not always for 'Oriented sampling'), soaked in 80% ethanol for 15 minutes and then transferred into a tube of fresh 80% ethanol for preservation (Hall et al. 2001). The tubes were labelled with date, collector, host species, stages of larvae, wound location and capture location (village, district, regency, and province) and kept at -20°C. All samples were identified using a published suite of diagnostic morphological characters (Hall 2008).

Seasonal dynamics of myiasis cases in Kediri regency, East Java

Kediri regency was chosen for a seasonal study because it is in an endemic area for traumatic myiasis, with a semi-intensive husbandry system, and the local veterinary clinic had a veterinarian who was prepared to collect data throughout a four-year period. The veterinarian and his two paramedic assistants monitored and controlled animal diseases across five districts (Kandat, Ngadiluwih, Ringinrejo, Kras, and Wates), and they recorded traumatic myiasis cases on their animal health reports to the regional livestock agency each calendar month, January 2006-December 2009.

Kediri regency (Figure 1) is located near the centre of East Java province, with 26 districts and 344 villages. Its agricultural system is mixed crop-livestock farming, typically with traditional semi-intensive livestock husbandry, for which animals are released from a stable (beside the farmer's house) c. 9 am and then returned to the stable c. 4 pm. No commercial farms, typically with >100 livestock and involved in short-term fattening programmes, were included in our sample. In the period 2006 - 2009 the maximum and minimum annual temperatures were 33°C and 22°C, respectively, and the area received an average rainfall of 1000-2000 mm per annum (BMKG 2017).

Mitochondrial DNA typing

The DNA extraction technique was performed according to (Chomczynski et al. 1997) using the DNAzol® kit (Invitrogen Corp., Carlsbad, CA, U.S.A.). Successfully extracted DNA samples were dissolved in 1x Tris-EDTA solution and stored at -20ºC until analysis.

The primers used in this study were synthesized by MWG Biotech (UK) Ltd (Milton Keynes, U.K.). The 3' terminal end of the mitochondrial gene *cytochrome b* (*cyt b*) (761 base pairs (bp) of the CB fragment plus primers) was amplified using the primer pair CB1-SE (5' TATGTACTACCATGAGGACAAATATC 3') and PDR-WR04 (5' ATTTCACGCTCATTAACT 3').

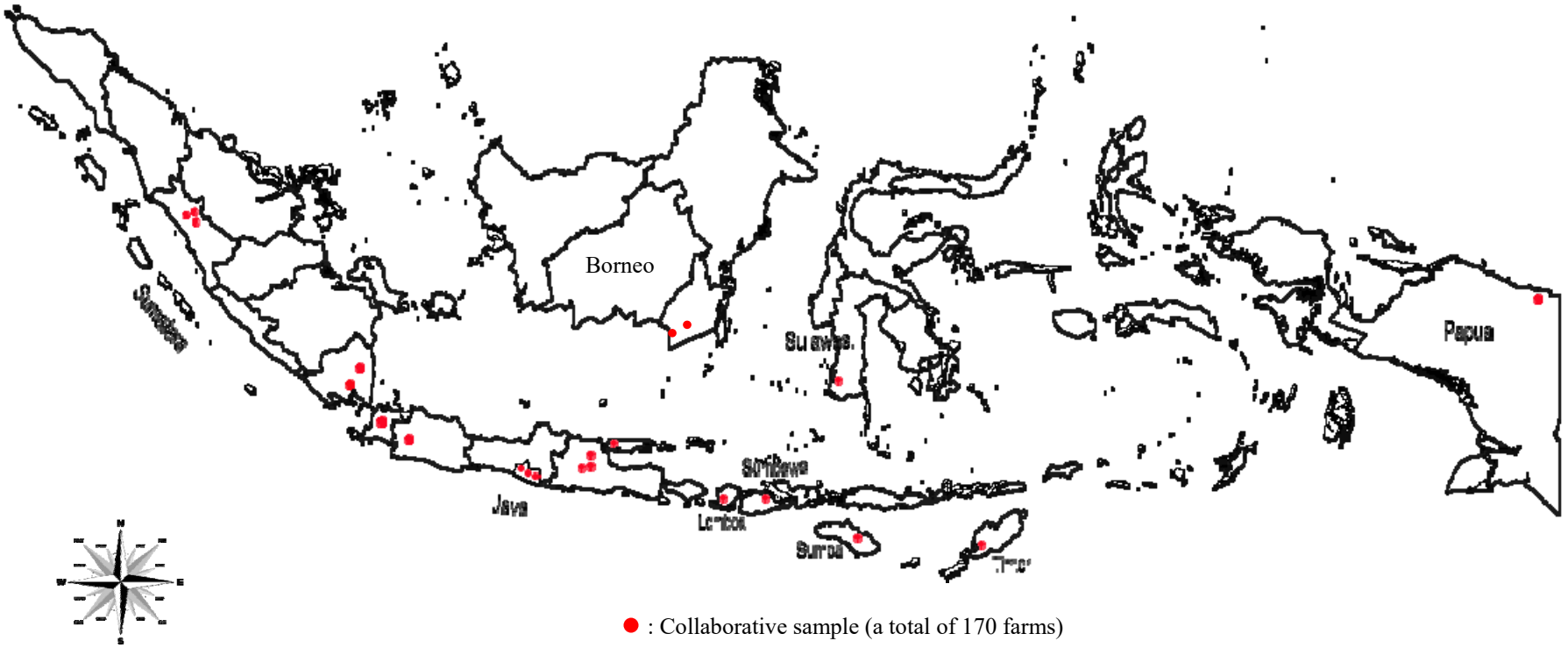

Figure 1. Map of OWS cases found in different regions of Indonesia.

The primers used in this study were synthesized by MWG Biotech (UK) Ltd (Milton Keynes, U.K.). The 3' terminal end of the mitochondrial gene *cytochrome b* (*cyt b*) (761 base pairs (bp) of the CB fragment plus primers) was amplified using the primer pair CB1-SE (5' TATGTACTACCATGAGGACAAATATC 3') and PDR-WR04 (5' ATTTCACGCTCATTAACT 3').

PCR conditions in the present study were optimized for dried specimens and those preserved in 80% ethanol (Ready et al. 2009; Wardhana et al. 2012), so that all could be properly analyzed (Hall et al. 2009a; 2009b). For this *cyt b* fragment (CB), the PCR conditions were commenced by a 'hot start' at 80°C, followed by an initial 3 min. denaturation step at 94°C, then 5 cycles of denaturation at 94°C for 30 seconds, annealing at 40°C for 30 seconds, ending with an extension step at 72°C for 90 second. This was followed by 35 cycles of denaturation, annealing and extension, where running parameters were those above except for a substituted annealing temperature of 44°C. The PCR reaction was finished by a final extension step of 72°C for 10 minutes and subsequent holding at 4°C. Sanger sequencing of each DNA strand used the primers CB1 and PDR-WR04, following the protocol of Ready et al. (2009).

Statistical analysis

All data were entered into a Microsoft Excel 2008 spreadsheet by date, location, host species, sex, age, and wound site. Categorical data were analysed by a Yates corrected χ^2 (Chi Square) test in Epi Info version 6.04d (Dean 1996) to test the effect of host species, host gender and wound site on the number of infestations. Continuous data, such as number of cases in different districts, were analysed with Unistat v. 6.0 and ANOVA in Stat-View v. 5.0. The results were considered significantly different at the 95% probability level ($P<0.05$).

RESULTS AND DISCUSSION

Predominance of OWS in traumatic myiasis wounds of livestock in Indonesia

The OWS fly was found to be the predominant species responsible for traumatic myiasis (255/260; 98.1%) in all collections from 2002-2009 (Table 1). *Musca* species (Muscidae) accounted for the other cases (5/260; 1.9%), with all found in rectum wounds of chickens as part of the 'oriented sampling'. These rare cases were not considered in any further analysis.

Pre-2002 cases were discussed by Wardhana et al. (2014), and again the OWS fly predominated from the first time this species was definitively recorded (Kraneveld & Pettinga 1949). The earliest case from 1926, reported by Kraneveld & Schaaf (1937), is now believed to have been caused by *Booponus intonsus* (Calliphoridae). Only OWS cases are considered further in the current report.

Geographical distribution of OWS in Indonesia

Historical records of OWS in Indonesia were reported and discussed by Wardhana et al. (2014), but they did not include a comprehensive table of all distribution records by province and regency because their report focused on ecological niche modelling based only on presence records that could be geo-referenced. The three kinds of surveillance carried out from 2002-2009 produced a total of 255 traumatic myiasis cases caused by the OWS fly in livestock from the western end to the eastern end of Indonesia. Table 1 includes these 255 cases from 22 regencies on 9 islands, which consist of records from one regency on Kalimantan (Tanah Laut), 5 regencies on Sumatra (Agam, Mungka, Payakumbuh, Lampung, Seputih Rahman), 9 regencies on Java (Banten, Sukabumi, Gunung Kidul, Klaten, Sleman, Blitar, Jember, Lamongan, Kediri), one regency on Madura (Bangkalan), one regency on Lombok (Mataram), one regency on Sumbawa (Sumbawa Besar), one regency on Sumba (East Sumba), one regency on Timor (Kupang), one regency on Sulawesi (Makassar) and one regency on New Guinea (Jayapura).

Larvae of *C. bezziana* were successfully collected from 12 regencies by 'collaborative sampling' and 'continuing sampling', which yielded 76 of the 255 cases (Table 2). The total livestock numbers on the 170 farms visited were recorded, allowing for an estimate of minimum prevalence rates of OWS on the two major hosts: cattle 0.3% (54/17359) and goats 3.2% (22/696).

'Oriented sampling' generated 179 cases. However, on the larger islands, veterinary clinics recorded no OWS cases in two out of two locations on Borneo (compared with cases discovered in one location investigated more actively by 'collaborative sampling' and 'continuing sampling'), no OWS cases in three out of three locations on Sumatra (compared with cases discovered in all 5 locations investigated more actively), and some OWS cases in one out of three locations on New Guinea (no locations investigated more actively) and, in marked contrast, many OWS cases in 8 out of 10 locations on Java. Table 1, based on 255 cases from widespread locations throughout the Indonesian archipelago, together with Table 3, based on 341 cases from Kediri, represent the most comprehensive record of myiasis cases from Indonesia to date.

Seasonal distribution of OWS in Kediri regency

A total of 341 traumatic myiasis cases was recorded in the five districts of Kediri province during 'collaborative sampling' and 'continuing sampling' 2006–2009 (Table 3). These cases were additional to the 255 mentioned previously (Table 1). The annual total was highest in 2007 (29.6% of the overall total) and lowest in 2008 (16.1%). The highest number of myiasis cases was found in Kandat district (66.6% of the overall total) followed by Ngadiluwih (27.6%). The other districts (Kras, Ringinrejo, Wates) had very few cases, with none reported in 2008.

The mean monthly abundance of myiasis cases in the five districts (based on the mean percentage of the annual total in each month, to enable comparison of years) was relatively stable, fluctuating between 5-13% of the annual total each month (equivalent to a mean of 5-11 cases per month) (Figure 2). There was variation from year to year, as shown by the standard error (SE) bars, but cases were found in all months with a peak occurring in December-January. If data from December and January were excluded, then in each year except 2006, and also for the combined data (2006-2009), the monthly percentages for February to November did not differ significantly from an expectation of an equal number of cases in each month (χ^2 = 10.5-14.5, df = 9; P = 0.10-0.31).

Table 1. Number of OWS cases found by 'collaborative sampling', 'continuing sampling' and 'oriented' sampling in different regions of Indonesia

Province	Island	Regency/City	Species		Total
			C. bezziana	*Musca sp*	
South Borneo	Borneo	Tanah Laut	2	-	2
		Agam	28	-	28
West Sumatra		Payakumbuh	1	-	1
	Sumatra	Mungka	1	-	1
		Seputih raman	1	-	1
Southeast Sumatra		Lampung	11	2	13
Banten		Banten	28	-	28
West Java		Sukabumi	2	-	2
		Klaten	5	-	5
Central Java		Sleman	2	-	2
	Java	Gunung Kidul	41	-	41
		Kediri	69	-	69
		Blitar	4	-	4
East Java		Lamongan	1	-	1
		Jember	17	1	18
	Madura	Bangkalan	1	-	1
West Nusa Tenggara	Lombok	Mataram	10	-	10
	Sumbawa	Sumbawa Besar	14	-	14
East Nusa Tenggara	Sumba	East Sumba	4	-	4
	Timor	Kupang	1	2	3
South Sulawesi	Sulawesi	Makassar	8	-	8
West PNG	PNG	Jayapura	4	-	4
Total			255	5	260

Table 2. Seventy-six OWS cases discovered by collaborative sampling and continuing sampling in Indonesia and livestock numbers on the affected farms

Year	Province	Island	Regency	Total Farm	Total population		Number of cases	
					Cattle	Goat	Cattle	Goat
2005	East Java	Java	Kediri	32	105	15	2	1
	West Nusa Tenggara	Sumbawa	Sumbawa Besar	16	41	67	0	9
		Lombok	Mataram	23	34	103	4	8
	East Nusa Tenggara	Sumba	East Sumba	7	20	0	0	0
	South Sulawesi	Sulawesi	Sidrap	2	7003	0	8	0
	West Sumatra	Sumatra	Agam	12	40	0	4	0
2007	Banten	Java	Banten	10	3107	300*	25	1*
	Lampung	Sumatra	Seputih rahman	2	130	3	0	1
			Lampung	25	498	56	0	0
	South Borneo	Borneo	Banjar	25	737	0	0	0
2009	West Sumatra	Sumatra	50 Kota	1	0	2	0	1
			Payakumbuh	3	38	150	0	1
			Agam	9	31	0	7	0
	Banten	Java	Banten	3	5575	0	4	0
Total				170	17359	696	54	22

*=sheep

Hosts and wound sites of OWS

Based on the 255 cases discovered by all three types of sampling throughout Indonesia, myiasis occurred both in livestock and dogs (Table 4). The two most commonly infested hosts were cattle, 65.5% (167/255) followed by goats, 22.34% (57/255). The data also showed that the umbilicus and vulva were the most common wound sites among the hosts, in 23.1% (59/255) and 16.5% (42/255) of cases, respectively. As expected, the majority of umbilical myiasis cases was found in calves 79.7% (47/59), while vulval myiasis was found mostly in adult cattle, 71.4% (30/42). Other common wound sites were legs (12.6%, 32/255) and hooves (10.6%, 27/255). Of the cases specifying a gender, the number in female hosts (55.4%, 124/224) was significantly higher than in males (44.6%, 100/224) ($\chi^2 = 4.43$, df = 1, $P = 0.1088$).

Similarly to the 255 more widespread cases, the two most frequently recorded wound sites in Kediri during the investigation of seasonality (Table 3) were the vulva (25.8%, 88/341), mainly of cows after calving, and umbilicus of newborn calves (25.2%, 86/341). Unlike in the more widespread survey, where legs were found to be a major wound site (associated especially with poor stabling in Banten), in Kediri leg infestations were grouped in the bottom three (0.6%). However, as in the widespread survey, hoof infestations were common, being the only other site with more than 10% of records (14.4%, 49/341).

Mitochondrial typing of OWS flies from wounds

Of the 520 specimens from Indonesia typed for *cyt b* haplotype (Wardhana et al. 2012), 69 (13.3%), and 107 (20.6%) larvae came from wounds sampled for epidemiological investigations (Tables 3 and 4) in the regencies of Banten and Kediri, respectively (Table 5). Each larva was characterized by only one *cyt b* haplotype, which is consistent with maternal inheritance of the mitochondrial genome in flies. All haplotypes contained just one long open reading frame (ORF) with the expected stop codon, and so none was identified as a pseudogene that might be inherited chromosomally. The predominant haplotypes in each regency were haplotype CB_bezz06 of sub-lineage 2.2 and haplotype CB_bezz14 of sub-lineage 2.3, which together characterized 89.8% and 86.9% of the specimens from Banten (26 wounds) and Kediri (107 wounds), respectively (Table 5).

Most of the wounds in Banten (Table 6) and Kediri (Table 7) contained only one larval instar (73.1% and 55.8%, respectively), but some wounds contained different combinations of two larval instars (15.4% and 32.6%, respectively) or all three instars (11.5% and 11.6%, respectively). The proportion of these three types of wounds was not significantly different (Chi-square P>0.05) between the Banten sample with larvae typed for *cyt b* and the larger sample of 255 wounds from widespread Indonesian locations (Table 8), which comprised wounds with single instars (76.5%), two instars (18.4%) or three instars (5.1%). However, the proportion of these three types of wounds was significantly different (Chi-square P<0.05) between the *cyt b*-typed sample from Kediri and the larger sample of 255 wounds. This difference between Banten and Kediri remained if the analysis included only the wounds on cattle (25 and 26 wounds from Banten and Kediri, respectively) and not those on other hosts (just one wound on a sheep in Banten; but 15 wounds on goats and two wounds on dogs in Kediri) (Table 6 and 7). In Kediri, the ratio of wounds on different hosts was 26 cattle:15 goats:2 others, which was significantly different (Chi-square P < 0.05) to the ratio for the more general results, namely 167 cattle:57 goats: 31 others (Table 4). Any associations between *cyt b* haplotypes (or sub-lineages) and hosts were not analysed statistically, because the samples were temporally mixed. However, it can be noted that the two predominant haplotypes from sub-lineage 2.2 (CB_bezz06) and sub-lineage 2.3 (CB_bezz14) were found on each of the main hosts (cattle and goats). These hosts were also infested by each of the predominant haplotypes from sub-lineage 2.1 (CB_bezz02, CB_bezz12) on Sumatra.

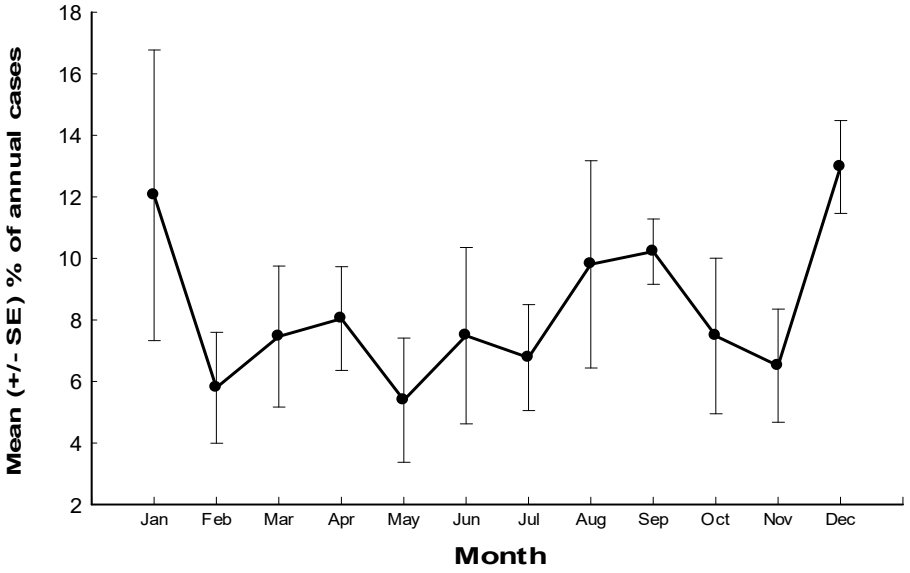

Figure 2. Mean monthly OWS cases as a percentage of the annual total (± SE) in the Kediri regency, 2006-2009.

Table 3. Number of OWS cases recorded by collaborative sampling and continuing sampling in five districts of Kediri regency

Districts	Years				Total
	2006	2007	2008	2009	
Kandat	59	66	33	69	227
Ngadiluwih	18	29	22	25	94
Wates	2	3	-	2	7
Ringinrejo	2	2	-	1	5
Kras	5	1	-	2	8
Total	86	101	55	99	341

Table 4. Distribution of myiasis wounds by livestock host and wound location among the 255 OWS cases discovered by 'collaborative sampling', 'continuing sampling' and 'oriented sampling' in Indonesia

Wound location	Gender of host			Host species										Total
	F	M	NR	Cattle		Sheep		Goat		Buffalo	Horse	Dog	Pig	NR	
				Cattle	Calf	Sheep	Lamb	Goat	Kid						
Umbilicus	20	28	11	4	47	-	3	3	2						59
Vulva	42	-	-	30	2	1	1	7	1	-	-	-	-	-	42
Leg	6	15	11	26	-	-	1	-	1	1	3	-	-	-	32
Hoof	18	8	1	17	-	-	-	9	-	-	-	-	1	-	27
Tail	7	3	-	-	-	5	-	2	3	-	-	-	-	-	10
Udder	9	-	-	8	-	1	-	-	-	-	-	-	-	-	9
Neck	3	4	2	5	1	-	-	-	-	-	2	1	-	-	9
Prepuce	-	8	-	5	-	-	-	2	1	-	-	-	-	-	8
Muzzle	2	6	-	6	2	-	-	-	-	-	-	-	-	-	8
Rectum	4	3	-	-	1	3	-	3	-	-	-	-	-	-	7
Mouth	2	5	-	-	3	-	-	4	-	-	-	-	-	-	7
Ear	4	1	-	2	-	-	-	2	-	-	-	1	-	-	5
Abdomen	-	7	1	-	-	-	-	5	2	-	-	1	-	-	8
Horn	2	2	-	1	-	-	-	3	-	-	-	-	-	-	4
Hip	1	3	-	-	1	-	-	3	-	-	-	-	-	-	4
Back	-	3	1	-	-	-	-	1	-	-	-	3	-	-	4
Eye	2	1	-	2	1	-	-	-	-	-	-	-	-	-	3
Upper Head	-	2	-	1	-	-	-	-	-	-	-	1	-	-	2
Thorax	1	-	-	-	-	-	-	1	-	-	-	-	-	-	1
Scapula	-	1	-	1	-	-	-	-	-	-	-	-	-	-	1
N R	1	-	4	1	-	-	-	2	-	-	-	-	-	2	5
Total	124	100	31	109	58	10	5	47	10	1	5	7	1	2	255

Comment: NR : Not Recorded

Table 5. Frequencies of mitochondrial cyt b haplotype in larvae of the OWS fly sampled from livestock wounds in Banten and Kediri regencies

Mitochondrial *cyt b* typing		Banten sample		Kediri sample	
Haplotype CB_bezz	Sub-lineage	Number of larvae	Proportion, or haplotype frequency	Number of larvae	Proportion, or haplotype frequency
06	2.2	35	0.507	66	0.617
25	2.2	2	0.029	0	-
26	2.2	2	0.029	0	-
27	2.2	2	0.029	0	-
37	2.2	0	-	3	0.028
14	2.3	27	0.391	27	0.252
15	2.3	0	0.000	2	0.019
22	2.3	1	0.015	0	-
36	2.3	0	-	9	0.084
Total larvae		69		107	
Total wounds		26 [a]		43 [b]	

[a] See Table 6, [b] see Table 7

Table 6. Different complements of mitochondrial c*yt B* haplotypes (CB_Bezznn) in larvae [A] of the OWS fly from single wounds on 26 livestock in Banten regency

Haplotype complement	Larval instars with haplotype complement in each wound			Numbers of each host with such wounds			Total wounds
	L1	L2	L3	Cattle	Sheep (goats)	Dogs	All hosts
			Single larval instar in each wound				
HC 2	- [b]	06	-	1	0	0	1
HC 3	-	-	06	10	1	0	11
HC 4	-	-	14	5	0	0	5
HC 6	-	-	06, 14	1	0	0	1
HC 8	-	-	14, 22	1	0	0	1
Sub-totals				18	1	0	19
			Multiple larval instars in each wound				
HC 9	06, 14	06	-	1	0	0	1
HC 13	-	06	25	1	0	0	1
HC 16	-	14	14	2	0	0	2
HC 20	06	06	06	1	0	0	1
HC 22	14	14	06, 14	1	0	0	1
HC 23	27	06, 14	26	1	0	0	1
Sub-totals				7	0	0	7

[a] Two larvae of each instar were individually typed from each wound; b Not present
L1 1st instar; L2 2nd instar, L3 3rd instar

Table 7. Different complements of mitochondrial *cyt b* haplotypes (CB_bezzNN) in larvae [a] of the OWS fly from single wounds on 43 livestock in Kediri regency

Haplotype complement	Larval instars with haplotype complement in each wound			Numbers of each host with such wounds			Total wounds
	L1	L2	L3	Cattle	Goats	Dogs	All hosts
				Single larval instar in each wound			
HC 1	06	- [b]	-	1	0	0	1
HC 2	-	06	-	3	0	0	3
HC 3	-	-	06	8	6	0	14
HC 4	-	-	14	1	3	0	4
HC 5	-	-	36	1	0	0	1
HC 7	-	-	06, 36	0	1	0	1
Sub-totals				14	10	0	24
				Multiple larval instars in each wound			
HC 10	14	06	-	1	0	0	1
HC 11	06	-	06	1	0	0	1
HC 12	-	06	06	1	0	0	1
HC 13	-	06	14	2	0	0	2
HC 14	-	06	14, 36	1	1	0	2
HC 15	-	06	36	0	1	0	1
HC 17	-	14	06	2	0	0	2
HC 18	-	15	06	1	0	0	1
HC 19	-	36	36	1	0	0	1
HC -	-	37	NR [c]	0	0	1	1
HC -	-	NR	06	0	1	0	1
HC 21	06	14	14	0	1	0	1
HC 24	37	06	06	0	0	1	1
HC -	NR	NR	14	1	0	0	1
HC -	NR	06	14	0	1	0	1
HC -	NR	06	06, 14	1	0	0	1
Sub-totals				12	5	2	19

a Two larvae of each instar were individually typed from each wound; b Not present ; c No result
L1 1st instar; L2 2nd instar, L3 3rd instar

Multiple OWS infestations of the same wound

Multiple infestations of a wound were detected by the presence of more than one larval instar and/or more than one *cyt b* haplotype of the OWS fly. Each larva contained only one identified *cyt b* haplotype, and only two larvae of each instar were usually typed from each wound. Therefore the detection of all haplotypes in a wound was not guaranteed, and the results provide a minimum estimate of multiple infestations.

Considering only the presence of single or multiple larval instars, not of any *cyt b* haplotype diversity, infestations with third instars alone formed the most common type of myiasis recorded among 255 wounds from widespread locations (65.5%), followed by multiple infestations with both second and third instar larvae (15.7%) (Table 8). When comparing the proportions of occurrence of single- and multi-instar infestations, there were no significant differences (Chi-square $P>0.05$) between the six regencies with large

numbers of OWS infestations (Agam and Lampung on Sumatra; Banten, Gunung Kidul, Kediri and Jember on Java; Table 1), between the predominant hosts (cattle, goats and sheep) (Table 8), or between the five most infested sites on hosts (umbilicus, vulva, leg, hoof and tail) (Table 4). However, there were insufficient data to perform multivariate analyses to identify any co-variation between geographical region, host species, wound location and season.

Furthermore, multiple infestations are likely to be underestimated because, in well sampled locations, *cyt b* typing indicates that secondary infestations can occur over periods short enough to produce wounds occupied by only a single instar, i.e. with two or more haplotypes present in the same instar. This was the case for three wounds with only third instar larvae but two *cyt b* haplotypes (two in Banten, one in Kediri) and five multi-instar wounds with two *cyt b* haplotypes found in larvae of the same instar (three in Banten, two in Kediri) (Table 6 and 7).

There was no evidence for any association of a *cyt b* haplotype or sub-lineage with primary or secondary infestation. Based on 23 fully characterized multi-instar wounds (Table 6 and 7), only haplotypes of one sub-lineage were found in all the instars from 8 wounds; 6 wounds contained the oldest instars with haplotype CB_bezz06 (or another haplotype of sub-lineage 2.2) in the absence of haplotype CB_bezz14 (or another haplotype of sub-lineage 2.3); the reverse was true for 7 wounds; and, both sub-lineages were present in the oldest instars from two wounds.

Discussion

Geographical distributions of myiasis caused by fly species and their genetic lineages

The OWS fly was the primary cause of traumatic myiasis of livestock in Indonesia, and no larvae of the facultative parasite *Chrysomya megacephala*, the sister species, were found in any of the wounds, despite this species being widespread in the country (Wardhana et al. 2012). Only a very few cases of chicken myiasis caused by *Musca* species were recorded. Myiasis of poultry by species of Calliphoridae is generally rare, reported for *Lucilia sericata* [on turkeys in Iraq (Al-Khalidi & Shareef 1985) and on geese in Hungary (Farkas et al. 2001)] and for *Cochliomyia macellaria* and *Phaenicia* species [on chickens in Panama (Bermúdez et al. 2007)] and the OWS fly [on chickens in India (Jeyathilakan et al. 2011)].

In the present study, more larvae and myiasis cases were found in Kediri, East Java (27.1% of 255) than in any other location. However, we believe that the results were correlated with sampling intensity and local veterinarians' interest in myiasis and their enthusiasm for surveillance, rather than with case prevalence or the OWS fly's population density. Most infestations discovered 2002-2009 came from local veterinary clinic services participating in the 'collaborative sampling' and 'continuing sampling' schemes, such as in Kediri, rather than our 'oriented sampling' scheme, which was ineffective outside Java. All three sampling schemes

Table 8. Numbers of myiasis wounds on different livestock hosts that contained single and multiple larval instars among the 255 OWS cases discovered by 'collaborative collections', 'continuing collections' and 'oriented collections' in Indonesia

Host species	Larval instars [a]							Total
	L1	L2	L3	L1 + L2	L1 + L3	L2 + L3	L1 + L2 + L3	
Cattle	6	13	105	2	3	30	8	167
Sheep	1	1	12	-	-	1	-	15
Goat	3	4	38	2	-	6	4	57
Horse	-	-	5	-	-	-	-	5
Buffalo	-	-	1	-	-	-	-	1
Dog	-	-	3	-	-	3	1	7
Pig	-	-	1	-	-	-	-	1
NR [b]	-	-	2	-	-	-	-	2
Total	10	18	167	4	3	40	13	255

[a] L1 1st instar; L2 2nd instar, L3 3rd instar
[b] NR Not recorded

were variants of passive case detection, which cannot be relied upon (Vreysen 2005) to demonstrate changes in the population density of screwworm in an area. It is more likely to be a reflection of reporting efficiency and the level of farmer cooperation.

The OWS fly in Indonesia is characterised by mitochondrial DNA haplotypes grouped into four sub-lineages, with the Asian mainland sub-lineage 2.1 predominating on Sumatra in the west, sub-lineages 2.2 and 2.3 predominating on Java, sub-lineage 2.2 predominating on the smaller islands of Wallacea (Nusa Tenggara and Maluku), and sub-lineage 2.4 predominating on New Guinea in the east (Wardhana et al. 2012). The samples analysed in the current report reflected this pattern.

Seasonal and environmental distribution of OWS myiasis infestations

The surveillance data from 2006-2009 showed numbers of reported myiasis cases in the range of 55-101 per year in five districts of Kediri and demonstrated that traumatic myiasis is a major problem for small farmers in this regency of East Java. Kandat and Ngadiluwih districts showed a higher number of cases of myiasis than other districts (Table 3). These two districts are only about 2 km from the animal clinic where the data was collected, making it more likely that traditional farmers would report their livestock problems to the veterinarians. Wates, Ringinrejo and Kras were approximately 10 km from the clinic and the larger farmers in those districts reported their livestock problems to private veterinarians who do not maintain an accurate record of myiasis cases. Therefore the myiasis problem is likely to be significantly greater than suggested by the reported data.

Even though cases could be found in every month during the four-year study period, there was a slight seasonal trend for myiasis cases to peak in Kediri in December-January (Figure 1), during the rainy season (http://www.worldweatheronline.com). This finding is consistent with what is known about the seasonality of OWS in other Asian regions. Although the peak of myiasis cases might occur in different months in different geographical regions, the peak months everywhere experience similar climatic conditions, i.e. moderately high temperature and humidity (Sutherst et al. 1989). In this way, the highest number of OWS cases in Iraq and Oman occurred in the cooler months of December-March (Al-Taweel et al. 2000; Spradbery 1992; Siddig et al. 2005), and cases in Saudi Arabia usually peak in March-May when there is higher rainfall (Alahmed et al. 2006). However, even if temperature and humidity are major determinants of the seasonality of the OWS fly, many other environmental factors can directly or indirectly influence its distribution, such as human population density, cattle density, elevation and tree cover (Wardhana et al. 2014).

Seasonality is unlikely to have significantly affected our investigations into host preferences and wound locations of OWS (see the next section), because most of the widespread cases came from the larger islands, where seasonal differences in climate are small and similar to those in Kediri on Java (Wardhana et al. 2014).

Hosts of traumatic myiasis and wound sites

The predominant host animals attacked by *C. bezziana* in Indonesia in this study were cattle (65.5%) followed by goats (22.4%) (Table 2 and 4). However, this in itself does not necessarily imply that cattle are more susceptible to myiasis or are the preferred host because the numbers of livestock at risk of infestation in the same herds/flocks need to be taken into account. Where available, these data suggest that goats are at much greater risk of OWS infestation than cattle, with 5.3% (21/396) of goats infested compared to 0.31% (54/17,359) of cattle in the same geographical areas (Table 2). The goat data were skewed by the relatively high level of infestation of goats in West Nusa Tenggara (17/170 = 10%; Table 2). Clearly more extensive surveys of OWS myiasis prevalence are required in Indonesia to definitively report on OWS host preferences and the risks of myiasis infestation by OWS to different hosts in different regions of the country.

The major wound sites recorded in this study were the umbilicus of calves and the vulva of cattle, associated with calving. Calving is clearly a major risk period for infestation by the OWS fly and a period in which farmers should regularly check their livestock. The same is true for NWS fly, *Cochliomyia hominivorax*, but wound sites are also clearly related to environmental factors. Hence in the Yucatan Peninsula of Mexico, the most frequently observed sites of NWS infestations on cattle were related to vampire bat bite sites on the neck and shoulders, umbilicus sites being third numerous (Thomas 1987). Other common sites of myiasis in our study were the leg and hoof, possibly attributed to the poor condition of the stables: those with slippery and dirty floors caused some livestock to be wounded in their limbs or to develop foot-rot (Muharsini et al. 2010; personal observations in this study).

Hall et al. (2009a) noted that identification of wound sites is important in the treatment of myiasis because it indicates the best sites for the potential application of topical prophylactics for control of myiasis. Giving treatment preferentially to the hind

quarters of the host would assist the protection of organs such as the genitalia, anus, udder and umbilicus.

Multiple infestations

Mature, third instar larvae were those most frequently removed from wounds, suggesting that farmers generally neglected to check their livestock as regularly as they should or simply overlooked smaller wounds with immature larvae during the first 5-6 days after egg laying. In the current study, the proportions of livestock wounds with multiple larval instars were somewhat similar in cattle (42/167 = 25.7%) and goats (12/57 = 21.1%). A substantial number of wounds were found with two larval instars (18.4%, 47/255) and a smaller number with all three instars (5.1%, 13/255) in sites as varied as hoof, prepuce, vulva, umbilicus, muzzle, thigh, ear and head (Table 8). Infestations in host sites that are difficult to handle and inspect would encourage the occurrence of multiple infestations. For example, although only a few prepuce infestations were recorded, a high proportion had multiple-instar infestations (75%, 6/8). Multiple infestations are underestimated if based only on the presence of multiple larval instars, because mitochondrial DNA typing demonstrated that larvae of the same instar often originate from two or more mothers, which must lay their eggs within 1-36 hours of each other.

Overlooked economic importance of OWS

This study clearly demonstrated that cases of traumatic myiasis caused by the OWS fly are widespread in Indonesia and occur frequently, despite the relatively small number of cases reported in the literature and by veterinary services. One reason for this might be the low mortality associated with myiasis if treatment is timely. Myiasis is not classified nationally among the diseases of strategic importance, such as anthrax, brucellosis, rabies, foot and mouth disease and avian influenza. Therefore, myiasis treatments are left to livestock owners to apply. Nevertheless¸ if infested livestock are not treated appropriately they can die or suffer permanent disability, and so the economic effects on small farms, with small numbers of livestock, can be relatively severe. Although our survey was completed in 2009, the dangers of screwworm myiasis still persist in Indonesia, not only to wild and domesticated animals, but also to humans (Hidayat et al. 2016).

The Indonesian government has yet to analyse the economic impact of OWS infestations on the livestock industry, but the impact will be significant if case prevalences are similar to those in adjacent Malaysia. Grindle et al. (2001) showed that the total annual loss of livestock production due to myiasis infestations in Malaysia was around RM 18 million (US $4.7 million). Similarly, Nor (2002) estimated that the annual losses to the Malaysian livestock industry due to myiasis infestation were about RM 23 million (US $ 6 million). Taking into account the plans of the Malaysian government to expand animal production, the economic impact was projected to reach RM 32 million (US $ 8.5 million) in 2005 and RM 50 million (US $ 13.1 million) in 2010 (Grindle et al. 2001). These estimates are similar to those from an economic analysis of the impact of *Cochliomyia hominivorax* in Cuba (Grindle et al. 2001).

A cost-benefit analysis of OWS myiasis prevention and control is required, in order to assess the economic effects on national and international trade of livestock. The epidemiological data presented in the current report provide a useful baseline of the host and spatio-temporal distribution of OWS in Indonesia, on which to build a more robust picture of monthly transmission, in order to support a control or eradication programme using SIT as a component of Integrated Pest Management (IPM). Accurate prevalence and incidence data are required, based on standardised surveillance of randomized samples of livestock, because current case densities reflect variable surveillance activity. This makes it difficult for Indonesia to regularly submit surveillance data to the World Animal Health Information Database (WAHID).

CONCLUSION

The epidemiology of traumatic myiasis caused by the OWS fly in Indonesia is complex, with multiple infestations of wounds frequent. The plasticity in host selection demonstrated by all mitochondrial lineages of the OWS fly in Indonesia is clearly advantageous to this species, as is its lack of marked seasonality, both of which facilitate colonization of new areas and diverse hosts.

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   a. Lawrence TLJ, Fowler VR. 2002. Growth of farm animals. 2nd ed. New York (USA): CABI Publishing.
   b. Bamualim A, Tiesnamurti B. 2009. Konsepsi sistem integrasi antara tanaman padi, sawit, dan kakao dengan ternak sapi di Indonesia. In: Fagi AM, Subandriyo, Rusastra IW, penyunting. Sistem integrasi ternak tanaman padi, sawit, kakao. Jakarta (Indones): LIPI Press. p. 1-14.
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Umiyasih U, Antari R. 2011. Penggunaan bungkil inti sawit dan kopra dalam pakan penguat sapi betina berbasis limbah singkong untuk pencapaian bobot badan estrus pertama >225 kg pada umur 15 bulan. Prasetyo LH, Damayanti R, Iskandar S, Herawati T, Priyanto D, Puastuti W, Anggraeni A, Tarigan S, Wardhana AH, Dharmayanti NLPI, editors. Proceeding of National Seminar on Livestock Production and Veterinary Technology. Bogor (Indones): Indonesian Center for Animal Research and Development. p. 192-199.

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