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PREFACE

Starting volume 21 Number 1 2016 Indonesian Journal of Animal and Veterinary Sciences has been accredited by the Ministry of Research, Technology, and Higher Education with Decree Number 21/E/KPT/2018. This accreditation will last until Volume 25 Number 4 2020 (website: Sinta).

In this edition, volume 23 No 3, we proudly present articles from animal and veterinary sciences including feed and nutritive technology, and chemical residue. The articles published in this edition are: "Blood biochemical components and progesterone hormone on the day of estrus in crossbred cattle in Egypt"; "Effectiveness of bioactive combinations of several plant substances to inhibit the growth of *Escherichia coli* and *Salmonella* sp."; "Blood profile of implantation Stainless Steel 316L local implant material on rat femoral bone"; "Ultrasonographic and vaginal cytological diagnostics of the Queen"; "Aflatoxin M1 in fresh dairy milk from small individual farms in Indonesia; and "Nutrition quality and microbial content of buffalo, cow and goat milk from West Sumatera".

We extent high appreciation to all peer reviewers who make this journal academically high value. Hopefully, these articles would offer any benefit to readers and the end-users of technological innovation, and attract interests from scientists to contribute their papers to the Indonesian Journal of Animal and Veterinary Sciences.

Chief Editor

Bogor, September 2018

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Blood Biochemical Components and Progesterone Hormone on Day of Estrus in Crossbred Cattle in Egypt

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ABSTRAK

Mourad RS. 2018. Komponen biokimia darah dan hormon progesteron pada estrus sapi persilangan di Mesir. JITV 23(3): 103-111. DOI: http://dx.doi.org/10.14334/jitv.v23i3.1855

Kekurangan atau kelebihan mineral seperti P, Cu dan Zn dikaitkan dengan kesuburan subnormal dan kondisi anoestrus. Penelitian ini dilakukan di unit veteriner pada 18 betina sapi persilangan yang dipilih secara acak pada saat estrus (kelompok estrus) dan sebagai control dipilih 14 ekor sapi persilangan dari sapi-sapi yang baru beranak sekitar 6 - 12 jam (kelompok kontrol). Tujuan penelitian ini adalah untuk mempelajari komponen biokimia dan hormon progesteron pada hari estrus dari sapi persilangan di Mesir, informasi dari penelitian ini akan digunakan untuk mengkonfirmasi terjadinya estrus untuk meningkatkan persentasi keberhasilan perkawinan. Dalam plasma darahnya, konsentrasi semua komponen biokimia dan konsentrasi progesteron pada ternak yang estrus lebih tinggi daripada sapi kontrol kecuali globulin. Dari Sapi control didapatkan kosentrasi Mg, Ca / P ratio, Co, Cu, Zn, Se, dan Mo yang lebih tinggi dari pada sapi estrus. Di sisi lain, konsentrasi plasma darah Na, K, Ca, P, Mn, dan Fe lebih tinggi pada sapi estrus daripada pada sapi kontrol. Konsentrasi progesteron sapi estrus lebih rendah saat musim panas daripada disaat musim dingin. Pada sapi estrus, semua unsur makro plasmanya lebih tinggi disaat musim dingin daripada dimusim panas kecuali rasio K, Ca dan Ca/P. Selanjutnya pada sapi estrus, semua unsur mikro plasma darahnya lebih tinggi disaat musim dingin dibandingkan saat musim panas kecuali Zn, Mn, Se, dan Fe.

Kata Kunci: Sapi, Glukosa, Kolesterol, Mineral, Progesteron

ABSTRACT

Mourad RS. 2018. Blood biochemical components and progesterone hormone on day of estrus in crossbred cattle in Egypt. JITV 23(3): 103-111. DOI: http://dx.doi.org/10.14334/jitv.v23i3.1855

Deficiencies or excess minerals such as P, Cu, and Zn are associated with subnormal fertility and anoestrus conditions in cows. This study was conducted in a veterinary unit in Menufiya, Egypt. Eighteen head of crossbred cows were randomly selected at random at estrus time (estrus group) and as a control 14 head of crossbred cows were selected from newly-calving dams at about 6-12 hours after calving (control group). The aim of this study was to use the components of biochemistry and progesterone hormone on estrus day of crossbreeding cattle in Egypt. The information from this study will be used to confirm estrus time to improve mating percentage. In blood plasma, the concentrations of all biochemical components and progesterone concentrations in estrus animals are higher than control cows except globulin. The results showed that blood plasma from control animal obtained Mg, Ca / P ratio, Co, Cu, Zn, Se, and Mo concentration is higher than estrus cattle. On the other hand, blood plasma concentrations of Na, K, Ca, P, Mn, and Fe are higher in estrus cattle. The progesterone concentration of estrus cattle is lower during summer than in winter. In estrus cows, higher plasma macro-elements were found in winter than in summer except for K, Ca and Ca/P ratio. On the other side of estrus cattle, all trace elements of blood plasma are higher in winter than in summer except Zn, Mn, Se, and Fe.

Key Words: Cattle, Glucose, Cholesterol, Minerals, Progesterone

INTRODUCTION

Reproduction is one of the critical pillars of dairy production. Many dairy herds do not achieve their targets for reproductive performance and incur a substantial economic opportunity cost. Reproductive management has received increased attention in recent years as new technologies and programs have been developed to aid dairy managers in efficiently breeding cows and heifers (Widmar & Wolf 2008). Despite the fact that postpartum uterine disease is only one component of reproductive performance, and that it generally has secondary importance to insemination efficiency (LeBlanc 2005), it has traditionally occupied a substantial amount of veterinarians' attention. Effective breeding management is a crucial tool to enhance the reproductive and productive performance of dairy cattle. Failure to detect and correctly interpret signs of estrus can contribute to a significant loss in the dairy industry (Barr 1975; Britt 1985).

Visual heat detection programs were relatively more sensitive to labour costs than synchronization programs. In addition, past reproductive performance was found to affect the determination of which reproductive program was optimal. Farms that had attained high levels of labour efficiency for visual heat detection in the past had less incentive to adopt a synchronization program than farms with less efficient visual heat detection (Widmar & Wolf 2008). Although, this visual observation of animals for signs of estrus is a standard method of estrus detection across the globe, in developed countries objective tests, are used to confirm estrus (Rao et al. 2013), including measuring serum/plasma or milk progesterone (P4) levels, and to successfully breed cattle (Mekonnin et al. 2017).

Minerals are the essential nutrients bearing a significant role in the animal reproduction because their excess or deficiency produces a detrimental effect on the performance of livestock. Trace elements including Cu, Co, Zn, Fe, Se, I, Mo, Mn and certain macro-elements like K, Ca, Na, Cl, P have been found to be essential for healthy livestock growth (Underwood 1981). Trace elements may function as cofactors, as activators of enzymes, or as stabilizers of secondary molecular structure (Valee & Wacker 1976). Deficiency or excess of minerals like P, Cu and Zn have been associated with subnormal fertility and anoestrus conditions (Moddie 1965). The aim of this study was to use the components of biochemistry and progesterone hormone on estrus day of crossbred cattle in Egypt. The information from this study will be used to confirm estrus time to improve mating percentage.

MATERIALS AND METHODS

The present study was carried out in veterinary units in Menufiya, Egypt during one year from May 2013 to April 2014. Eighteen head of crossbred of 4-9 years old cows that have 2-6 parity experience with 335 to 556 kg LBW were randomly selected at estrus time (estrus group) and as a control 14 head of crossbred cows were selected from newly-calving dams at about 6-12 hours after calving (control group). Cows were fed rations consisted of a commercial concentrate feed mixture (60% yellow corn, 15% wheat bran, 23% soybean meal, 1% Na CL and 1% mineral mixtures), Egyptian clover (Trifolium alexandrinum) and rice straw, while others were fed bran and green clover and rice straw. Animals were milked twice daily morning and evening milking. Blood samples were collected from the jugular vein of all cows. Blood samples were collected into clean heparinized test tubes and transported to the laboratory, centrifuged at 3000 rpm for 20 minutes and blood plasma was carefully separated and stored frozen at –20°C until the time of chemical analyses. Blood plasma was carefully digested by adding 10 ml concentrate H_2SO_4 and two drops of H_2O_2 to 1 ml of blood plasma and heated. The digested sample was diluted with distilled water at a ratio of 1:50. The concentration of macro- (Na, K, Mg, and Ca) and micro- (Mn, Cd, Se, Co, Cu, Mo, Fe, and Zn) elements were determined in blood plasma using an atomic absorption spectrophotometer (UNICAM 929 AA®). A standard ICP-OES (Perkin-Elmer, Optima 2000 DV®) analyzer system was used for the determination according to (Oser 1965). Progesterone was estimated in both plasma and milk by the radioimmunoassay (RIA) technique using the coated tube kits according to Haynes et al. (1980) and Blight & White (1983). The kit was purchased from Institute of Isotopes Co. LTD. Budapest and was labelled with I 125. The tubes were counted using computerized Gamma Counter (Packard Instrument Company®). The standard curve of P4 ranged between 0.0 and 37.7ng/ml. All reagents were equilibrated to room temperature. Then duplicate tubes were labelled for each of total counts (T), non-specific binding (NSB) zero standard (standard 1=B0), standards (S2-6), control (C) and either milk or plasma samples (S). The reagents and samples were mixed thoroughly before used, then 50 µl from each of standards, control, and samples were inserting into the adequately labelled tubes. Thereafter 100 µl of tracer solution and 100 µl of antiserum were pipetted into all tubes except T and NSB, which were antiserum free. All tubes were vortex mixed thoroughly for 2-5 seconds except T. The tubes were incubated for 2hours at room temperature (20-28°C), and then they were placed on a separate tube rack. A bottle containing magnetic immunosorbent (MIS) was shacked and swirled gently until homogeneity then 500µl was added to each tube except T. All tubes were vortex mixed thoroughly and incubated for 15 minutes at room temperature. The bound fraction was magnetically separated. The rack was attached to the magnetic separator base, and the MIS particles were settled for 5 minutes. The supernatant was poured off and discarded. The tubes were placed on a pad of absorbent tissue and allowed to drain for 2 minutes. The bound fraction was also separated by centrifugation for 15 minutes at 1500xg. The radioactivity of all tubes was counted preferably not less than 60 seconds. Data obtained were statistically analyzed using the computer program of SAS (2004) (Descriptive statistics, ANOVA and Duncan test was used to determine the significant differences among means at α=0.05).

RESULTS AND DISCUSSION

Progesterone and biochemical components

The concentration of blood biochemical components including cholesterol (CH), glucose (G), total protein

Table 1. Concentrations of P4 and blood biochemical components in control and on the day of estrus in crossbred cattle

Items	Case	Mean ± SE	Min	Max
Progesterone concentration P4 (ng/ml)	Control	$0.50{\pm}0.01^{a}$	0.42	0.55
	Estrus	$0.58{\pm}0.01^{a}$	0.50	0.66
Blood biochemical components:				
Cholesterol (mg/dl)	Control	$74.74{\pm}0.71^{b}$	72.59	78.65
	Estrus	$217.93{\pm}12.11^{a}$	121.95	273.17
Glucose (mg/dl)	Control	$65.25{\pm}0.14^{a}$	6.38	7.68
	Estrus	$58.78{\pm}3.1^{b}$	33.93	71.43
Total protein (mg/dl)	Control	$6.08{\pm}0.09^{b}$	5.48	6.35
	Estrus	$6.77{\pm}0.25^{a}$	4.86	8.00
Albumin (mg/dl)	Control	$1.17{\pm}0.05^{b}$	0.90	1.33
	Estrus	$4.80{\pm}0.22^{a}$	3.07	6.46
Globulin (mg/dl)	Control	$5.29{\pm}0.19^{a}$	4.71	6.61
	Estrus	$1.97{\pm}0.15^{b}$	0.47	3.13
A/G Ratio	Control	$0.22{\pm}0.58^{b}$	0.12	1.49
	Estrus	$2.43{\pm}0.05^{a}$	1.09	2.02

Control (n=14), Estrus (n=18)
Significant different ($P<0.05$) marked by different superscript letter in the same row for each blood biochemical component

(TP), albumin (AL), globulin (GL), AL/GL ratio and progesterone concentration (P4) and those at time of estrus are depicted in the Table (1). Results show that concentrations of all blood biochemical components and (P4) concentrations at estrus were higher than control cows except (GL).

In the control cows that the animal is just calving the level of P4 was not significantly different than in the estrus cows. It is often assumed that a larger corpus luteum will produce more progesterone and generate higher circulating plasma concentrations. Corpus luteum weight and plasma progesterone concentration increased between day 5 and day 8 of the estrus cycle. During the early stage of corpus luteum development, a relationship between size and progesterone is present, by day 8 of the cycle, the size of the corpus luteum is no longer of importance in determining circulating progesterone concentrations (Mann 2009).

Díaz et al. (1988) reported that the P4 profiles showed no differences among groups. In Holstein P4 levels ranged from 0.5 ng/ml plasma on the day of estrus (Day 0) to 5.1 ng/ml at the luteal phase peak (Day 13). In Brahman, P4 levels ranged from 0.5 ng/ml on Day 0 to 9.2 ng/ml on Day 13. In Carora-type and crossbred females, P4 levels ranged from 0.5 ng/ml, on Day 0, to 13.7 ng /ml on Day 12 and 8.8 ng/ml on Day 13.

On the other hand, Mondal & Prakash (2003) reported that in Sahiwal cows with overt estrus, plasma progesterone concentrations during periestrus, early luteal, mid-luteal and late luteal phase were 0.40±0.02, 0.74±0.10, 1.94±0.22, and 0.63±0.16 ng/ml, respectively and the corresponding values in cows with silent estrus being 0.47±0.03, 0.94±0.08, 1.39±0.13, and 0.95±0.19 ng/ ml, respectively. The overall plasma progesterone levels in cows that exhibited overt estrus were 1.23±0.99 ng/ml as against 1.08±0.09 ng/ml in silent estrus. It was concluded that progesterone levels were lower ($P>0.05$) in cows that exhibited silent estrus compared to overt estrus.

Blood glucose appears to be one of the key nutrients affecting cyclicity in farm animals, and a minimum level of 60-40 mg/ml is required to maintain the physiological processes of the body (Duke 1970). According to Dowine & Gelman (1976), low blood glucose may be associated with infertility. Dutta et al. (1988) reported significantly lower serum glucose level in anoestrus than normally cycling animals. El-Azab et al. (1993) reported significantly higher serum protein in cyclic cows than the noncycling ones. However, Tegegne et al. (1993) found an inconsistent trend. Qureshi (1998) reported higher blood urea level in anoestrus animals than those resuming cyclicity within 45 days postpartum. Burle et al. (1995) reported the lowest serum concentration of cholesterol in anoestrus than in cycling cows. Significantly variable reports were available on the level of these biochemical constituents. Lyubestsky (1997) reported higher values of total serum protein in endometriotic buffaloes and cows compared to cyclic buffaloes and cows, respectively. However, Burle et al. (1995) reported the significantly higher value of total serum protein in cyclic cows.

Plasma glucose concentration was significantly (P<0.01) higher in estrus animals that in anoestrus animals, Singh & Singh (2006). In the anoestrus group of animals, the mean value of cholesterol was insignificantly higher than the mean value in estrus animals. The mean values of the total serum protein of crossbred heifers for the estrus group as well as the anoestrus group were found in the normal range. An optimum level of total protein in blood serum is essential for the expression of estrus sign in cows. Deficiency of protein intake in cows can cause infertility, and altered hormonal balance caused by elevated blood urea nitrogen levels also may decrease fertility and reproductive (Amin 2014).

Optimum concentrations of blood glucose and proteins induce estrus cyclicity via hypothalamo-hypophyseal system (Tandle et al. 1998). Detrimental effects of protein metabolites (blood urea nitrogen) may occur not only at different stages of oocyte development but also during fertilization and blastocyst formation (Jorritsma et al. 2003). High plasma glucose and proteins were recently reported in cycling dairy cattle compared to non-cycling (Kumar et al. 2010).

Saied (2017) reported that the means of total protein was 6.5±1.2 g/dL in crossbred cattle in Egypt. Dezfouli et al. (2013) reported that the means of total protein and glucose were 7.38±0.39 g/dL and 6.17±0.40 mg/dL, respectively in Holstein dairy cows and crossbreeds in the Tehran.

Macro-and micro-elements

The concentration of blood macro- and micro-elements in plasma of control cows and those with reproductive disorders are shown in the Table (2, 3). In control cows: Mg, Ca/P ratio, Co, Cu, Zn, Se, and Mo were higher than in the estrus cows. On the other hand, the blood concentrations of Na, K, Ca, P, Mn, and Fe was higher in estrus than in control cows.

Meanwhile, Small et al. (1997) reported that serum Ca, Na, S, Cu, Fe, Zn, and Mn did not differ (P>0.05) between first service conception groups, but sodium (Na) and copper (Cu) were higher (P<0.05) at estrus than at day 21, especially in nulliparous heifers. Concentrations of serum magnesium (Mg) were low (<0.74 mmol/L) and potassium (K) high (>4.5 mmol/L) in heifers and cows but in nulliparous heifers only, serum magnesium (Mg) and potassium (K) were lowest (P<0.05) at estrus for those that conceived. Serum boron (B) concentrations were generally higher (P<0.05) in animals that conceived especially on day 21 when boron (B) concentrations were higher (P<0.05) than at estrus. Serum phosphorus (P) concentrations were high (>3.5 mmol/L), but highest in animals that conceived especially on day 21. It is concluded that phosphorus (P) and boron (B) may be dietary factors limiting first service conception in beef cows fed conserved forage.

Macro-elements such as Ca, P, Na, Mg, K, S, and Microelements such as Cu, Fe, Zn, Mn, and Se are essential nutrients that function in the regulation of cellular metabolism and when deficient in ruminant diets have caused reproductive problems (Hidiroglou 1979; ARC 1980; Underwood 1981).

Saied (2017) reported that the means of Na, K, Ca, and P were 140.04±0.85 mEq/l, 5.20±0.22 mEq/l, 9.11±0.2 mg/dl, and 3.39±0.20 mEq/l, respectively in crossbred cattle in Egypt. Dezfouli et al. (2013) reported that the means of Na, K, Mg, Ca, and P were 140.600±3.050 mmol/L, 4.340±0.321 mmol/L, 1.99±0.19 mg/dL, 9.76±0.76 mmol/L, and 6.17±0.40 mg/dL, respectively in Holstein dairy cows and crossbreeds in the Tehran.

Table 2. Blood macro elements in normal and on the day of estrus in crossbred cattle

Case	Macro	Mean±SE (mg/dl)	Min	Max	Macro	Mean±SE (mg/dl)	Min	Max
Control	Na	142.13±0.25[b]	139.32	144.93	Ca	5.46±0.16[b]	6.16	16.80
Estrus		164.95±7.04[a]	101.1	208.3		13.65±0.54[a]	4.60	6.40
Control	K	4.21±0.10[b]	3.68	4.72	P	1.92±0.04[b]	1.61	2.00
Estrus		24.66±1.46[a]	6.35	35.28		5.57±0.30[a]	3.18	7.90
Control	Mg	4.46±0.07[a]	3.89	4.65	Ca/P Ratio	2.87±0.14[a]	2.32	3.78
Estrus		2.86±0.19[b]	1.63	4.51		2.53±0.14[b]	1.74	3.82

Control (n=14), Estrus (n=18)
Significant different (P<0.05) marked by different superscript letter in the same row for each macro-elements

Table 3. Blood micro-elements in control and on the day of estrus in crossbred cattle

Case	Micro	Mean±SE (µg /dl)	Min	Max	Micro	Mean±SE (µg /dl)	Min	Max
Control	CO	0.89±0.11[b]	0.49	1.54	Se	10.46±0.24[a]	9.14	12.02
Estrus		3.18±0.28[a]	1.30	4.61		0.44±0.03[b]	0.22	0.59
Control	CU	1.26±0.14	0.66	1.84	Fe	0.35±0.02[b]	0.20	0.45
Estrus		1.04±0.16	0.09	2.02		3.42±0.57[a]	1.10	7.77
Control	Zn	5.46±0.09[a]	4.91	5.72	Mo	0.59±0.01[a]	0.58	0.66
Estrus		0.74±0.07[b]	0.20	1.08		0.04±0.002[b]	0.02	0.05
Control	Mn	0.82±0.047[a]	0.65	1.13	Cd	0.14±0.002[b]	0.13	0.15
Estrus		0.89±0.37[a]	0.04	6.70		0.75±0.08[a]	0.19	1.44

Control (n=14), Estrus (n=18)
Significant different (P<0.05) marked by different superscript letter in the same row for each micro-elements

Concentrations of minerals in blood serum or plasma are generally related to intake but are influenced by sex, breed, age, and reproductive status, e.g., pregnancy or lactation (ARC 1980; Underwood 1981). Differences in serum mineral concentrations between first service conception groups at estrus/first service may have been indicative of an influence on ovulation and subsequent development of the corpus luteum. Plasma levels of P as low as 1.56 mmol L–1 have not affected fertility in dairy cows (Brodison et al. 1989).

Concentrations of K in forage were above the NRC (NRC 1984) since blood K has been shown to be related to dietary K (Fisher et al. 1994). Serum Ca concentrations were normal and did not differ between first service conception groups. Dietary concentrations of Ca were higher than NRC (NRC 1984) recommended minimum allowances for heifers and cows. However, the rise in serum Estradiol at estrus would have stimulated an increase in serum Ca (Horst 1984). A deficiency of Mg was not expected, yet serum Mg was <0.745 mmol/L which has defined hypomagnesia in cattle (ARC 1980). Others have shown that supplemental Cu and Mg improved fertility in dairy cows even though plasma concentrations of Cu and Mg were similar among supplemented and un-supplemented groups (Ingraham et al. 1987).

Serum Na was higher at estrus than on day 21, especially in nulliparous heifers and cows. In nulliparous heifers, serum S was also higher at estrus than on day 21 especially in animal (Small et al. 1997). Similarly serum Fe, Zn and Mn concentrations were Control (Puls 1981) and did not differ among the first service groups. Barui et al. (2015) reported among the minerals; zinc was found to play the most vital role to cause repeat breeding condition in the farm animals. A significant difference in the levels of calcium, phosphorus, magnesium, and iron was observed in the case of serum minerals. Mean phosphorus level in the anoestrus animals was lower than the suggested critical level of 4.5 g/100ml (Kunj et al. 2006). Saied (2017) reported that the means of Cu and Fe were 10.39±0.36 and 140.5±0.15 mEq/l, respectively in crossbred cattle in Egypt.

Effect of the season on blood biochemistry

The effect of season on the concentration of blood biochemical components at estrus time is depicted in the Table (4). This study represents that progesterone concentrations tended to be higher on day of estrus in winter than in summer but not significantly different and these results disagree with Rao et al. (1982) who reported that progesterone concentrations on the day of estrus and also in the luteal phase of the cycle were significantly higher (P≤0.01) in the colder seasons than in the hot and dry and hot and humid seasons. Meanwhile, Roussel et al. (1977) found a significantly higher level of progesterone in monthly samples for cows in the hot season. Also, all blood biochemical components were higher on the day of estrus in winter than in summer.

Cholesterol concentration in estrus cows was higher in winter than in summer. In line, the result was reported in Nguni cattle resulted. It was concluded that Nguni cattle had lower cholesterol in the hot-wet season than crossbreds and energy deficits mostly occurred during the late cool-dry season on the sweet rangeland (Mapiye et al. 2010). Glucose concentration in estrus cows was higher in winter than in summer. The decrease in plasma glucose and plasma cholesterol during the hotter period was reported Abeni et al. (2007).

Table 4. Seasonal variations of blood biochemical components in control and on the day of estrus in crossbred cattle

Items	Control		Estrus	
	Summer	Winter	Summer	Winter
P4 conc.$_{(ng/ml)}$	0.49±0.01[a]	0.50±0.01[a]	0.54±0.02[a]	0.59±0.01[a]
Cholesterol$_{(mg/dl)}$	74.62±2.02[a]	74.78±0.78[a]	168.97±16.86[b]	231.92±12.65[a]
Glucose$_{(mg/dl)}$	7.59±0.09[a]	7.15±0.16[a]	38.91±4.89[b]	64.46±2.09[a]
Total protein$_{(g/dl)}$	6.27±0.08[a]	6.02±0.12[a]	5.51±0.55[b]	7.13±0.19[a]
Albumin$_{(g/dl)}$	1.32±0.01[a]	1.13±0.06[a]	3.86±0.36[b]	5.07±0.22[a]
Globulin$_{(g/dl)}$	4.75±0.02[a]	5.44±0.23[b]	1.65±0.20[b]	2.06±0.19[a]
Alb/Glob Ratio	148.75±0.40[a]	146.65±0.67[a]	1.43±0.02[a]	1.43±0.06[a]

Control (n=14), Estrus (n=18)
Significant different (P<0.05) marked by different superscript letter in the same row between each group within the same blood biochemical component

Effect of season on macro- and micro-elements

The effect of season on macro- and micro-elements at estrus time is depicted in the Tables (5, 6). This study represents that in hot and cold season, Na, K, Ca, and P were higher on the day of estrus than Control cattle. On the other hand, Mg and Ca/P Ratio were lower on the day of estrus than Control cattle. Pearson et al. (1964) could not demonstrate a change in plasma Ca values of five mature cows when Ca estimations were performed daily during four estrus periods. Other workers, Bacih et al. (1969) reported that the total serum Ca of a Shorthorn cow, but not of a Jersey, fell about 2 mg per 100 ml at the time of estrus.

Minerals are involved in all living processes, either in the capacity of structural elements or as regulators of almost all metabolic processes (Bauman & Currie 1980). Apart from the above-indicated roles, minerals are essential for milk production of high-yielding cows. Consequently, their role assumes increasing importance. Homeostasis of calcium, phosphorus, and magnesium is primarily affected by the very same homeostatic mechanisms, and as a result, the changes in their concentrations are in most cases mutually linked. Calcium is the most pervasive mineral in an organism. The maintenance need of calcium is approximately 15.4 mg Ca/kg of body mass (Hansard et al. 1954). The need for lactation is approximately 1.23 g Ca/kg of the produced milk. Calcium absorption depends on its intake, and it is reduced when the animal takes more calcium in its food than it is needed.

The research about calcium level in dependence on the physiological condition and seasonal variations did not show the influence of the above-indicated factors on calcium values in cows (Yokus et al. 2006). However, Krnić et al. (2003) reported not only considerably lower calcium values in cows 1-5 days from calving but also hypocalcemia in lactating cows and in cows 5-10 days before calving. Lower bottom values of calcium are also reported by Olayemi et al. (2001). They found that 39% of cows had the calcium concentration < 2 mmol/L. The values of magnesium concentration in the literature vary widely such as 0.7–1.2 mmol/L (Merck 2003), 0.74–0.95 mmol/L (Kaneko et al. 2008; Radostits et al. 2000).

Nejra & Josip (2012) indicated a greater need of cows for phosphorus than that provided by plants contained in the animal diet, and this is a direct consequence of low values of this mineral in the soil composition. The age of animals also influences the value of phosphorus concentration. In other words, the phosphorus level increases with the age of animals (Roussel et al. 1982). Moreover, this study represents that in the hot season, Co, Mn, Fe, and Cd were higher on the day of estrus than Control cattle. On the other hand, Cu, Zn, Se, and Mo were lower on the day of estrus than Control cattle. Nejra & Josip (2012) have proved the influence of sampling season on the value of calcium concentration in lactating cows and those in dry period (Figure 1). In cows up to fifteen days from calving, the influence of sampling season on the value of calcium has not been proved. The calcium level depends on the feed so that the different botanical make-up of pastures, and grazing in general, may be a cause of significant differences established during various seasons of sample taking. Certain values of calcium concentration have shown significant differences depending on the reproductive cycle of cows. The highest value of calcium concentration has been established in cows in the dry period with samples taken in summer while lactating and postparturient cows have significantly lower values of calcium concentration in the same period. The mean calcium

Table 5. Seasonal variations of plasma macro elements on the day of estrus in crossbred cattle

Items (mg/dl)	Control		Estrus	
	Summer	Winter	Summer	Winter
Na	6.19±0.16[a]	6.24±0.19[a]	128.05±15.13[b]	175.49±5.46[a]
K	4.15±0.16[a]	4.23±0.13[a]	27.29±2.23[a]	23.91±1.75[b]
Mg	4.60±0.02[a]	4.42±0.08[a]	2.17±0.36[b]	3.06±0.19[a]
Ca	5.47±0.32[a]	5.46±0.19[a]	14.89±1.15[a]	13.29±0.59[b]
P	1.87±0.13[a]	1.94±0.04[a]	5.48±0.24[a]	5.60±0.39[a]
Ca/P Ratio	2.98±0.40[a]	2.84±0.15[a]	2.71±0.10[a]	2.49±0.18[b]

Control (N=14), Estrus (N=18)
Significant different (P<0.05) marked by different superscript letter in the same row between each treatment within the same Macro element

Table 6. Seasonal variations of microplasma elements on the day of estrus in crossbred cattle

Items (µg /dl)	Control		Estrus	
	Summer	Winter	Summer	Winter
Co	0.84±0.35[b]	0.91±0.11[a]	1.97±0.24[b]	3.53±0.30[a]
Cu	1.57±0.27[a]	1.18±0.16[b]	0.69±0.12[b]	1.14±0.19[a]
Zn	5.67±0.02[a]	5.40±0.11[b]	1.70±0.17[a]	0.74±0.07[b]
Mn	0.72±0.07[b]	0.85±0.06[a]	1.29±0.45[a]	0.78±0.46[b]
Se	10.14±0.15[a]	10.54±0.31[a]	0.29±0.05[a]	0.49±0.03[b]
Fe	0.35±0.01[a]	0.35±0.02[a]	6.35±0.94[a]	2.59±0.49[b]
Mo	0.61±0.03[a]	0.59±0.01[a]	0.03±0.002[a]	0.04±0.002[a]
Cd	0.13±0.001[a]	0.14±0.003[a]	0.38±0.04[b]	0.86±0.09[a]

Control (N=14), Estrus (N=18)
Significant different (P<0.05) marked by different superscript letter in the same row between each treatment within the same Microplasma element

level determined during summer in cows in the dry period was found to be higher (3.1±0.16 mmol/L) than those of the other published research findings (Kaneko et al. 2008; Merck 2003).

In this respect, all plasma macro elements were higher on the day of estrus in winter than in summer except K, Ca and Ca/P ratio. On the other hand, all plasma macro elements were higher on the day of estrus in winter than in summer except Zn, Mn, Se, and Fe. During the summer period, significantly lower levels of calcium were determined in cows immediately after calving. In interpreting the calcium level, we should not neglect the physiological hypocalcemia which occurs after calving, especially in dairy cows. If we make a comparison with the established reference values, we can notice hypocalcemia in many periparturient cows. However, these values are appropriate for the postpartum physiological condition (Oltner & Bergland 1983).

CONCLUSION

Mineral concentrations from plasma blood sample of estrus crossbred cattle were lower than control cows. The concentrations of all blood biochemical components in the estrus cows were higher than that of control cows except for P4 and globulin concentration. Control animal contained higher Mg, Ca/P ratio, Co, Cu, Zn, Se, and Mo than that of estrus cattle. On the other hand, control cows have lower Na, K, Ca, P, Mn, and Fe than estrus cows. Season affected macro-elements of estrus cows. In estrus cows, higher plasma macro-elements were found in winter than in summer except for K, Ca, and Ca/P ratio. On the other side of estrus cattle, all trace elements of blood plasma are higher in winter than in the summer except Zn, Mn, Se, and Fe.

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Effectiveness of Bioactive Combinations of Several Plant Substances to Inhibit the Growth of *Escherichia coli* and *Salmonella* sp.

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ABSTRAK

Pasaribu T, Sinurat AP, Wina E, Purwadaria T, Haryati T, Susana IWR. 2018. Efektifitas campuran bahan bioaktif beberapa tanaman dalam menghambat pertumbuhan bakteri *Escericia coli* dan *Salmonella* sp. JITV 23(3): 112-122. DOI: http://dx.doi.org/10.14334/jitv.v23i3.1851

Antibiotik imbuhan pakan (AGP) telah dilarang penggunaannya sebagai pakan tambahan di berbagai negara, sehingga diperlukan penggantinya. Sebuah penelitian *in vitro* telah dilakukan untuk mempelajari potensi kombinasi beberapa ekstrak tanaman dalam mencegah pertumbuhan bakteri pathogen yang secara alami terjadi pada saluran gastrointestinal unggas dan *in vivo* untuk mengevaluasi populasi *E. coli* dalam ileum, respon imun dan profil darah ayam. Campuran bioaktif ketiga tanaman (asap cair dari cangkang *Anacardium occidentaleor* CLS, ektrak tanaman *Phyllanthun niruti* L.(EM) dan ekstrak *Synzygium aromaticum* extract (EDC) diformulasikan dan dievaluasi kefektifannya dalam menghambat pertumbuhan bakteri *Escericia coli* dan *Salmonela sp*. secara *in vitro*. Campuran (KE) tersebut kemudian dilarutkan dalam tiga konsentrasi yang berbeda, yaitu: 100%KE; 50%KE dan 25%KE yang kemudian diamati kefektifannya dalam menghambat pertumbuhan *E. coli* dan *Salmonella sp.* menggunakan metode *microplate reader*. Dalam uji biologis, kombinasi bioaktif adalah pada konsentrasi 0,0625% CAM + 0,0625% EM + 0,0313% EDC. Perlakuan terdiri dari 8 jenis ransum, masing-masing terdiri dari 2 ulangan dan setiap ulangan terdiri dari 5 DOC. Pada akhir percobaan (35 hari), darah diambil dari 2 ekor ayam pada setiap replikasi. Hasil penelitian menunjukkan bahwa semakin tinggi konsentrasi campuran bioaktif (KE100), semakin tinggi pula kemampuannya dalam menghambat pertumbuhan bakteri *E. coli* dan *Salmonella sp*.. Campuran bahan bioaktif CAM, EM dan EDC lebih efektif dibandingkan antibiotik Zn-bacitracin dalam menghambat pertumbuhan *E. coli* dan *Salmonella sp*.. Konsentrasi optimal KE dengan kefektifan serupa dengan AGP adalah 25%. Dapat disimpulkan bahwa campuran CAM, EM dan EDC mampu menghambat partumbuhan bakteri *E. coli* dan bahkan mampu menghilangkan keberadaan bakteri *Salmonella* sp. Dalam pengukuran secara biologi, campuran CAM, EM dan EDC baik dalam bentuk ekstrak maupun bubuk dengan dosis tinggi, sedang maupun rendah tidak mempengaruhi bobot limfa, bursa fabricier dan profil darah. Campuran terbaik ekstrak CAM, EM dan EDC untuk menurunkan total populasi bakteri dan *E. coli* adalah dosis sedang yaitu 0,0625% (ekstrat) dan 0,625% (bubuk). Hal serupa juga berlaku pada penambahan bobot hidup pada penggunaan dosis bentuk ekstrak maupun bubuk yang tepat yang juga dapat menggantikan penggunaan antbiotik. Kombinasi CAM, EM dan EDC terbaik berpotensi sebagai pengganti AGP di dunia pakan unggas, khususnya ayam.

Kata Kunci: *Anacardium occidentale*, *Phylanthus niruri* L., *Synzygium aromaticum*, *E. coli*, *Salmonella* sp.

ABSTRACT

Pasaribu T, Sinurat AP, Wina E, Purwadaria T, Haryati T, Susana IWR. 2018 Effectiveness of bioactive combinations of several plant substances to inhibit the growth of *Escherichia coli* and *Salmonella* sp.. JITV 23(3): 112-122. DOI: http://dx.doi.org/10.14334/jitv.v23i3.1851

The use of antibiotic growth promoters (AGP) has been banned as feed additives in many countries, therefore the alternatives need to be found. An *in vitro* experiment was conducted to study the potential of combination of some plant extract to inhibit growth of pathogen bateria that normally occur in the poultry gastro intestinal tract and *in vivo* studies to evaluate the population of E. coli in the ileum, the immune response and blood profile of chicken. The combination of three plants bioactives (liquid smoke of cashew shells of *Anacardium occidentaleor* CLS, *Phyllanthus niruri* L. extract (EM), and *Synzygium aromaticum* extract (EDC) were formulated and evaluated for its effectiveness to inhibit growth of *Escherichia coli* and *Salmonella* sp. *in vitro*.The mixtures (KE) were then made in 3 different concentrations, i.e. 100 % KE, 50% KE, and 25% KE and studied their effectiveness to inhibit growth of *E. coli or Salmonella* sp. using microplate reader method. In biological assay, the bioactive combination was at a concentration of 0.0625% CAM + 0.0625% EM + 0.0313% EDC. The treatment consisted of 8 types of rations, each of it 2 replications and each replication consisted of 5 DOC. At the end of the experiment (35 days), blood was taken from 2 chickens at each replication. The results showed that the higher the concentration of the bioactive combination (KE100) the higher the ability to inhibit the growth of *E. coli* or *Salmonella* sp. The combination of bioactive substances CAM, EM, and EDC more effectively than Zn-bacitracin antibiotics to inhibit the growth of *E. coli* and *Salmonella* sp. The optimum concentration of KE with the similar effectiveness as the AGP was 25%. It was concluded that the combination of CAM, EM, and EDC was able to inhibit the growth of *E. coli* and even capable to eliminate the presence of *Salmonella* sp. In the biological assay, a combination of CAM, EM, and EDC either extract or powder form, high dose, medium or low does not affect the weight of the spleen, bursa fabricius, and blood profile. The best combination of CAM, EM, and EDC extracts to decreases the total bacterial population and E. coli was middle dose ie 0.0625% (extract) and 0.625% (powder). Likewise for live weight gain that was a good dose of extract or powder form can replace antibiotics. It was concluded that combinations of CAM, EM, and EDC had potential as a substitute for AGP in poultry feed, especially chickens.

Key Words: *Anacardium occidentale, Phylanthus niruri* L., *Synzygium aromaticum, E. coli, Salmonella* sp

INTRODUCTION

In general, antibiotic additives (AGPs / antibiotics growth promoters) are given to poultry by mixing them into feed or drinking water to increase production and prevent disease (Griggs & Jacob 2005). Giving AGP aims to minimize and even to reduce the population of pathogenic microbes in the intestine so that the nutrients contained in the gut are more dominantly absorbed. Pathogenic bacteria commonly found in the digest tracts of chickens and humans are *Escherichia coli* and *Salmonella* spp. In chickens, the bacteria are eliminated/killed by giving antibiotic supplementation so that the growth of livestock can increase up to 5-6% and the use of feed is more efficient up to 3-4% (Butaye et al. 2003). However, the use of antibiotics in livestock such as chickens can cause bacterial resistance to antibiotics. For example, Campylobacter and Salmonella against fluoroquinolone antibiotics and the third generation cephalosporins which is a constraint to the use of antibiotics in livestock (Noor & Poeloengan 2005). Many countries have banned the use of AGP, including Indonesia, as stated in Law No. 18 of 2009 juncto Law no. 14 of 2014. The ban on the use of antibiotics as a growth booster have been effective since January 1, 2018. Therefore, other materials such as AGP alternatives such as bioactive substances are needed. Several bioactive substances have been tested for their activity against pathogenic microbes *in vivo* (Lopez et al. 2012), but some are still *in vitro* test (Hoque et al. 2007).

Many local plants contain bioactive substances that function as antibacterial, antioxidant or antifungal such as cashew nut shells (*Anacardium occidentale*), meniran (*Phyllanthus niruri* L.), and clove leaf (*Syzygium aromaticum*). Several studies have been conducted for this purpose, such as the use of bioactive from Aloe vera, turmeric, *Curcuma zanthorrhiza* and *Morinda citrifolia* plants (Sinurat et al. 2004; Sinurat et al. 2009; Bintang et al. 2007).

Cashew (*Anacardium occidentale*) has pseudo-fruit waste and shell. When the shell extracted will be obtained liquid (oil) called biofat or Cashew Nut Shell liquid (CNSL). The waste of cashew nut shells is around 45-50%, with production of 137,496 tons in 2016, cashew shells is obtained around 61.8732 68.748 tons (SPI 2015-17). The bioindustry product of cashew nut shell consists of biofat, biosmoke and biochar (Saenab et al. 2016). Of these products, biofat (CNSL) has been widely studied, while biosmoke and biochar products from cashew shells have not been studied. Biosmoke (liquid smoke) is the result of decomposition process of cashew shells after extracted using hexane. The pyrolysis process is carried out with high heat without oxygen initiated by combustion and followed by total or partial oxidation of the main product (Bridgwater 2004). Liquid smoke from cashew shells can reduce the amount of *E. coli* in vitro (Sinurat et al. 2018), and suppress the growth of *Candida utilis* (Pasaribu et al. 2017).

Phyllanthus niruri L. (meniran) is a wild herb that is easy to grow in various geography. *P. niruri* L. can cure gastrointestinal disorders, and diarrhea because the content of bioactive substances is antimicrobial (Setyohadi et al. 2011). The compounds contained in the *P. niruri* L. ie alkaloids (0.122%), saponins (0.214%), tannins (0.040%), phenol (0.079%), glycosides (0.090%) (Gbadamosi et al. 2015). Water extract on meniran inhibits *E. coli*, *Staphylococcus aureus, Salmonella typhi* (Lestariningsih et al. 2015).

Syzygium aromaticum (cloves) contain the active compounds of phenol (eugenol, flavonoid, hydroxybenzoic acid, hydroxycinnamic acid and hydroxyphenyl propene (Cortés-Rojas et al 2014; Perez-Jiménez et al. (2010). Eugenol has been shown to have antifungal, antiseptic and insect repellents. Eugenol inhibits the growth of fungus by damaging the walls and permeability of cells resulting in impaired growth (Putri 2002). Clove water extract can inhibit the growth of *Escherichia coli, Staphylococcus aureus* and *Bacillus cereus* (Cortés-Rojas et al 2014).

Sinurat et al. (2018) have identified bioactive substances in 11 plants. Based on the results of the identification that liquid smoke from cashew nut shells (*Anacardium occidentale*) is potential as antibacterial, *Phyllanthus niruri* L.extract as an antioxidant, and clove leaf (*Syzygium aromaticum*) as an antifungal. With these three functions, it is hoped that the bioactive combination of the three plants has a higher inhibitory effect on the growth of *E. coli* and *Salmonella* sp.

This study aims to determine the inhibition power of the liquid smoke combination of cashew nut shells (*Anacardium occidentale*), *Phyllanthus niruri* L. extract and clove (*Synzygium aromaticum*) leaf extract on growth of *Escherichia coli* and *Salmonella* sp. *in vitro* as information for *in vivo* testing as a substitute for AGP in poultry.

MATERIALS AND METHODS

Preparation of extract and liquid smoke

The making of *Phyllanthus niruri* L. or clove leaf extraction and preparation of cashew nut shells was done at the laboratory of Ciawi Bogor, Indonesian Research Institute for Animal Production. The preparation of meniran or clove leaf extraction was done by inserting 1 gram of powdered meniran or clove leaves into the test tube, added with 10 mL methanol 70%, then put into ultrasonic for 30 minutes. Furthermore, centrifuged for 15 minutes at 2500 rpm, the top layer is taken with a pipette, this part is called as an extract of *P. niruri* L. (EM) or extract *Syzygium aromaticum* leaf (EDC). The production process of cashew shell liquid smoke was done in a small industry of liquid smoke and charcoal manufacture in Cinangneng Village, Bogor. Preparation of liquid smoke using cashew shells that have been previously extracted with hexane solution as described by Saenab et al. (2016).

In vitro inhibitory test

Test of inhibition of *Escherichia coli* and *Salmonella* sp carried out in the Laboratory of Microbiology Indonesian Research Institute for Animal Production (IRIAP) Bogor. *E. coli* and *Salmonella* sp used are collections of IRIAP cultures. Extracts of bioactive substances that have been prepared combined and observed for its effectiveness. The test dose refers to the optimum dose of each extract performed *in vitro* in the previous study (Sinurat et al. 2018). The test was done by microplate reader method to study the inhibitory power of bioactive substance, by inoculating *E. coli* or *Salmonella* sp into microplate which has 96 wells. Microplate reader is a tool to perform analysis of active compounds or microorganisms quickly based on turbidity measured with optical density (OD) using a spectrophotometer. The common detection modes measured in this tool are the absorbance and intensity of fluorescence (Petersen et al. 2014). Resistance to the growth of *E. coli* and *Salmonella* sp was observed by measuring the turbidity level on the microplate at a wavelength of 630 nm.

Preparation of microbial cultivation and plant bioactive substances

Escherichia coli or *Salmonella* sp culture with a concentration of 108 CFU/mL bacteria was made through its turbidity setting at the absorbance of 0.138 using a spectrophotometer at 620 nm wavelength. The liquid smoke of cashew shells (CAM), meniran extract (EM), clove leaf extract (EDC), and bacitracin (Bac) before being used for an inhibitory test, were first filtered using a filter membrane with 0.45 μm pores. CAM is made with a concentration of 0.25 by mixing 1 mL of pure CAM with 3 mL of aqueous. EM is made with a concentration of 0.25 by mixing 1 mL of pure EM with 3 mL of aqueous). EDC was made with a concentration of 0.125 by mixing 0.5 mL of pure EDC with 3.5 mL of aqueous. The three extracts were then mixed with a ratio of 1 : 1 : 1 (100%), i.e 1 ml CAM + 1 ml EM + 1 ml EDC is called 100% extract combination (KE100). Then, this combination was made in three concentration levels with 50% dilution (KE50) and 25% (KE25), by adding aquadest with a ratio of 1 : 1 and 1 : 3. As a comparison, a positive control (K +) is used Zn-Bacitracin at a concentration of 250 ppm. Blank is made by mixing aqueous with nutrient broth medium (NBM) without *E. coli* or *Salmonella* sp bacteria in microplate reader wells. Negative control (K-) is made by adding bacteria in aqueous solution. For K +, K- and other treatments, a well-filled nutrient broth medium was mixed with 140 μL bacteria.

In summary, the bacteria inhibitory treatment performed is as follows:

B = Blank (60 μL aquadest + 140 μL nutrient broth media/NBM)

K_ = Negative control (60 μL aquadest + 140 μL bacteria)

K+1 = Positive control (60 μL bacitracin 250 ppm + 140 μL bacteria)

K+2 = Positive control 2 (60 μL bacitracin 500 ppm + 140 μL bacteria)

KE100E = Combination KE100 extract (60 μL KE100 + 140 μL *E coli*)

KE50E = Combination of KE50 extract (60 μL KE50 + 140 μL *E coli*)

KE25E = Combination of KE25 extract (60 μL KE25 + 140 μL *E coli*)

KE100S = Combination of KE100% extract (60 μL KE100 + 140 μL *Salmonella* sp)

KE50S = Combination of KE50 extract (60 μL KE50 + 140 μL *Salmonella* sp)

KE25S = Combination of KE25 extract (60 μL KE25 + 140 μL *Salmonella* sp)

Data analysis was done by analyzing the pattern of Randomized Complete Design to compare the effect of treatment on the growth of *E. coli* and *Salmonella* sp.

This study has fulfilled the code of ethics no. Balitbangtan/Balitnak/A/ 03/2016.

Table 1. Combination dosage of the smoke of cashew shells (CAM), meniran extract (EM), And clove leaf extract (EDC) to be tested

Combination type	*A. occidentale* (%)	*P. niruri* L (%)	*S. aromaticum* (%)
Extract :			
High dose	0.125	0.125	0.0625
Medium dose	0.0625	0.0625	0.0313
Low dose	0.0313	0.0313	0.0157
Powder :			
High dose	0.125	1.25	0.625
Medium dose	0.625	0.625	0.313
Low dose	0.313	0.313	0.157
Zn-Bacitracin	0.05	0.05	0.05
Negative control	0	0	0

Exploration the effectiveness of combinations of bioactive substances in feed

Combination of plant bioactive: The combination of plant extracts consists of a combination of the liquid smoke of *Anacardium occidentale* (CAM) shell, *Phyllanthus niruri* L. (EM) extract, and *Syzygium aromaticum* leaf extract (EDC). A combination with various concentrations was conducted to test its effectiveness when mixed in the ration. Determination of concentration in ration was based on effective concentration to suppress *E. coli* and *Salmonella* sp growth in previous research *in vitro*, that is combination with concentration 0.0625% CAM + 0.0625% EM + 0.0313% EDC. For testing its effectiveness *in vivo* the concentration is increased twice for high doses and halved or to 50% for low doses. Testing the effectiveness of bioactive combination was also done using the powder form. The concentration of the ingredients in the form of flour uses the conversion 4 because 1 ml of the extract is obtained from 4 grams of flour. However, the CAM is not in the form of powder. Thus, the amount of each extract and powder added in the ration is presented in Table 1.

Chicken and management

Chicken used in this study was the broiler strain Ross maintained from day-old chick until 35 days. Feed and drinking water were given *ad libitum*. Chicken fed with the same composition, namely feed starter for 1-21 days old chickens and grower for chickens aged 22-35 days. The treatment consisted of 8 types of rations, each treatment consisted of 2 replications and each replication consisted of 5 DOCs. At the end of the experiment (35 days), blood was taken from 2 chickens on each replication. The basal ration composition was administered equally in all treatments, while the difference between treatments described with different levels of extract/powder combinations is described in Table 1. Body weight of the chicken weighed at the time of blood sampling (age 35 days). Then as much as 4 chickens from each treatment were euthanized to measure the weight of several internal organs (spleen and bursa fabricius). Intestinal contents were also taken for measuring the total amount of bacteria and the amount of *E. coli*. Calculation of total bacteria and *E. coli* was done 4 times in each treatment. Methods of calculating total bacteria and *E. coli* from the intestine were performed based on APHA procedure (2015).

Statistic analysis

Statistical analysis was performed by analysis of variance patterns Completely Randomized Design to compare between all treatments. Completely Randomized Design Factorial to compare between the extract and the powder form and between high, medium, and low doses. When variance analysis (ANOVA) is a significant difference between treatments at $P<0.05$ then continued with LSD test.

RESULTS AND DISCUSSION

The effect of combining CAM, EM, and EDC extracts on the growth of *E. coli* and *Salmonella* sp.

The effect of combining plant extracts on the growth of *E. coli* and *Salmonella* sp is shown in Figures 2 and 3. A 100% concentration of the combination of CAM, EM, and EDC more effective inhibits the growth of E. coli bacteria compared to 50% and 25% concentrations and treatment of Zn-bacitracin antibiotics. This result indicated that the combination of bioactive substances with high concentrations wasvery potent in inhibiting *E. coli* growth compared to concentrations of 50% and 25%. The concentration of a bioactive substance in medium (50%) and low (25%) was better inhibited the growth of *E. coli* and *Salmonella* sp than antibiotic zinc 2-methycardols 10-20% (Saidu et al. 2012; Setianto et al. 2009; Kumar et al. 2002). *P. niruri* L plants contain saponins, tannins, phenols, glycosides, flavonoids, and terpenoids (Gbadamosi et al. 2015), and *S. aromaticum* plants containing eugenol (Perez-Jiménez et al. 2010). Many types of active compounds present on these three types of plants (CAM, EM, and EDC) may have a role in the destruction of membranes, cell walls, and even cell nuclei of bacteria so that bacteria are unable to reproduce and eventually die (Harborne 1987; Turgis et al. 2009).

CAM has potential as a Gram-positive antibacterial (Himejima & Kubo 1991). The bioactive compound CNSL *A. occidentale* L. is able to penetrate the bacterial lipid bilayer membrane (Parasa et al. 2011), thus destroying the membrane bilayer. The same thing occurs to *E. coli* that cause *E. coli* defense to the outer environment to decrease, so *E. coli* can not develop. Where in *E. coli* replication occurs every 20 minutes. The bioactive substance in CAM has the potential to inhibit the growth of *E. coli* so that the population declines.

The bioactive substances present in *P. niruri* L at various concentrations are able to inhibit *E. coli* growth by destroying the membrane structure (Monte et al. 2014; Gbadamosi et al. 2015). *In vitro*, at 60% concentration, no bacterial colonies of S. dysenteriae (Munfaati et al. 2015) were found. The concentration of 10 mg/ml inhibits *E. coli* growth with an inhibitory zone of up to 29 mm (Gbadamosi et al. 2015). The concentration of *P. niruri* extract 0.0313 g/ml could inhibit the growth of *Edwardsiella tarda* bacteria (Sudarno et al. 2011). At concentrations of 1000 mg/mL also inhibited *E. coli*, *Staphylococcus aureus*, and *Salmonella typhi* (Ekwenye & Njoku 2006). Water extract on *P. niruri* with a concentration of 0.75% can result in a 23 mm clear zone in *E. coli* (Lestariningsih et al. 2015).

Eugenol as the most dominant bioactive substance in *S. aromaticum* plant with water extract at 3% concentration able to inhibit the growth of E. *coli*, *S. aureus* and *Bacillus cereus* (Cortés-Rojas et al. 2014). *S. aromaticum* extract with ethanol fraction has 18 mm inhibition zone, while extract with methanol fraction has 20 mm clear zone to *E. coli* (Pandey & Singh 2011). *S. aromaticum* extract at a concentration of 2000; 1500; and 1000 ppm showed each clear zone on *E. coli* about 13 mm; 9 mm, and 7 mm, and at the same concentration showed clear zone on *Salmonella typhi* 23 mm; 15 mm; and 10 mm (Kumar et al. 2014). The results of this study indicated that the combination of bacitracin (recommended dose for AGP). Therefore, for a combination of bioactive substances at a dose of 25% is sufficient to replace AGP. Several studies have shown that A. occidental plants contain active compounds of anacardic acid, cardanol, cardols, CAM, EM, and EDC could inhibit the growth of *E. coli* and *Salmonella* sp.

Figure 1 showed that the bioactive substances CAM, EM, and EDC at the lowest concentration have responded to inhibit the growth of *E. coli* is stronger than Zn-bacitracin antibiotics dose 500 and 250 ppm. The ability of a combination of CAM, EM, and EDC to inhibit the growth of *E. coli* at a low concentration (25%) match the ability of antibiotics. Therefore, in its application, the use of low concentrations can reduce the cost of bioactive substances.

Combinations of CAM, EM, and EDC with concentrations of 100%, 50%, and 25% indicated inhibition of Salmonella sp growth were similar (Figure 2). Similarly, López et al. (2012) CAM on broilers inhibits *Salmonella* sp growth in the digestive tract. In the study of Sinurat et al. (2018) also reported that CAM may inhibit the growth of *E. coli* and *Salmonella* sp. Research on the use of liquid smoke from cashew shells against *Salmonella* sp has not been done extensively so that the information is still limited.

P. niruri L significantly decreased *Salmonella* sp population in spleens infected with *Salmonella* sp (Sunarmo 2009), At concentration 31.25-62.50 mg/mL *P. niruri* L extract also decreased Salmonella sp (Ekwenye & Njoku 2006). Yusianti (2001) reported, *in vitro* use of *P. niruri* L extract at 0.6% concentration was able to inhibit the growth of *Salmonella pullorum*. Besides being a resistor of bacterial growth, *P. niruri* L extract was expected to increase the resistance of livestock body, because it has a high antioxidant ability (Sinurat et al. 2018).

The bioactive substances commonly found in plants are polyphenols, diterpene, alkaloids, and flavonoids (Rao et al. 2004). The delayed growth of *E. coli* and *Salmonella sp* is indicated because the bioactive substances of EM, CAM, and EDC are likely to form complexes with bacterial proteins through hydrogen bonds, consequently, the formation of nucleic acids and proteins in cells is inhibited. Turgis et al. (2009) stated that phenol (with-OH groups) can dissolve the lipids in the cell walls, thereby disrupting the cytoplasmic membrane performance and inhibit binding ATPase that cause cells to become lysis, as a result of bacterial growth is inhibited. Santoso (2008) reported, that the alkaloids contained in frangipani flowers (*Plumeria acuminata*) can suppress the growth of *S. dysenteriae* bacteria *in vitro*. From the results of *in vitro* assays, it is concluded that bioactive substances contained in the combination of EM, AM, and EDC are able to inhibit the total population of *E. coli* and *Salmonella sp*.

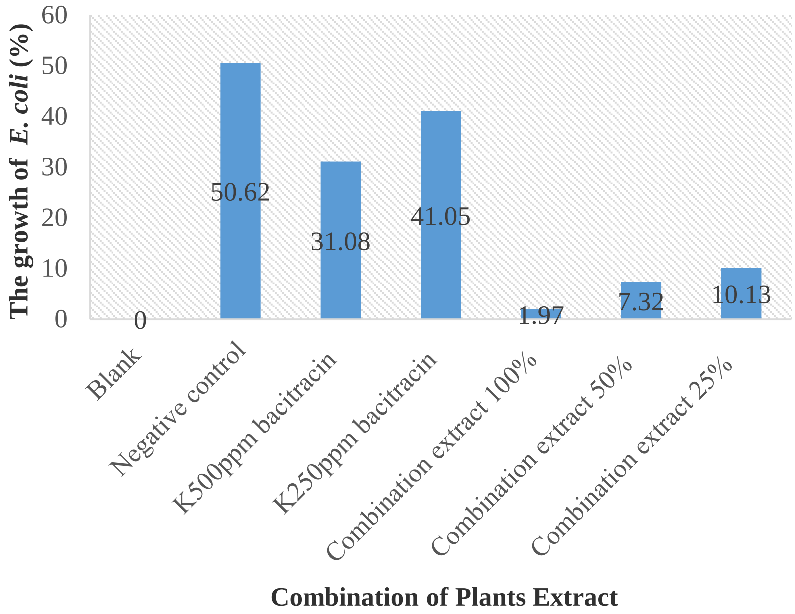

Figure 1. The effect of combining EM, CAM, and EDC extracts on *E. coli* growth.

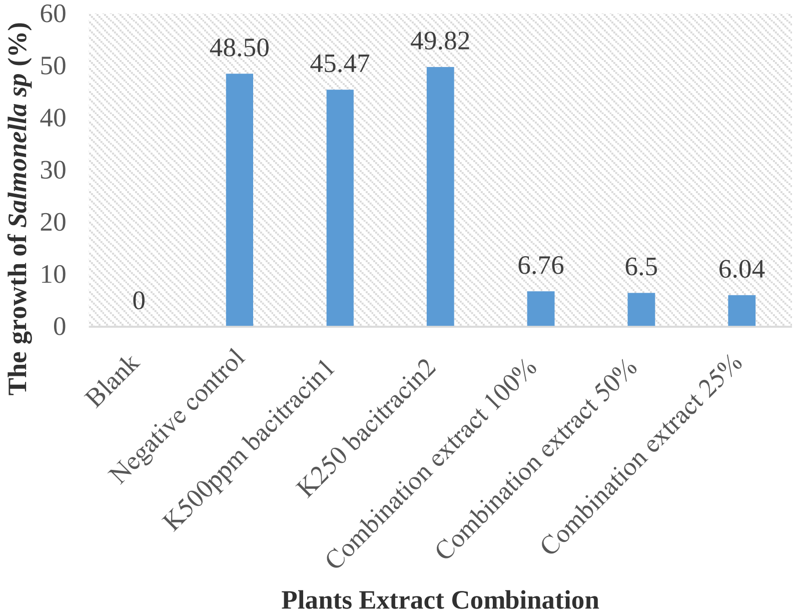

Figure 2. The effect of combining EM, CAM, and EDC extracts on *Salmonella* sp.

The effect of combination CAM, EM, and EDC on live weight, immune response, and blood profile

Body weight at the treatment of extract in medium and low dose, powder in high, moderate and low dose significantly ($P<0.05$) was significantly higher than negative control (Table 1). Combination of CAM, EM, and EDC in medium-dose at extract medium dose, powder in high, medium and low dose was not significantly different ($P>0.05$) with Zn-bacitracin treatment on live weight. But the treatment of medium and low dose extracts, high, medium and low-dose powder significantly ($P<0.05$) resulted in higher live weight than negative controls, which almost matched the antibiotic-fed chicken weight (Table 1). This result is only an early indication of the effect of the bioactive combination on chicken performance, since the number of tests and replication cattle used in this study is very limited. Tests using more numbers of livestock need to be done to determine the effect on livestock performance.

Giving combination CAM, EM, and EDC has the ability to replace antibiotics (Zn bacitracin), it was shown from the weight of life did not differ between them, while chicken negative control treatment showed lower body weight than other treatments. Attia et al. (2017) reported that a mixture of thyme extract 400 g, peppermint extract 300 g, green tea extracts 200 g, and licorice extract 100 g given to broiler chickens also showed improved performance compared to negative controls. The addition of a mixture of alfalfa leaf meal, cornflower, leaf senna and absinthe in broiler chicken feed also better than the control diet on enhances broiler growth (Khaligh et al. 2011). The mixture of some plants either in extract or flour form positively increases broiler weight, this also happens in combination CAM, EM, and EDC that can improve broiler performance. Bioactive substances of CAM such as phenol and anacardate act as antibacterials so that pathogenic bacteria in the intestine were eliminated and nutrients can be absorbed more optimally for growth needs. Khan (2010) reported that filantin, hypofilantin and flavonoids contained in EM act as a hepatoprotective agent (to prevent liver damage), thus metabolism in the liver such as protein synthesis for chicken growth is fulfilled. Giving eugenol as much as 100 mg/kg may improve the growth of chickens (Rychen et al. 2017). Likewise, eugenol contained in ED can increase chicken growth. Thus the combination of CAM, EM, ED has a positive synergistic effect on chicken performance.

Immune responses in chickens by administering a combination of plant bioactive

Statistical analysis of the relative weight of spleen and bursa fabricius and blood profile was not significant (P>0.05) between all treatments (Table 2). Spleen included in the secondary lymphoid functions to form granulocytes and erythrocytes. Relative spleen weights were not significantly different (P>0.05) among treatments, it was indicating the provision of a combination of CAM, EM, and ED at all doses did not affect the production of granulocytes, and erythrocytes in chickens. This indicates that when chickens are given a combination of CAM, EM, and EDC does not interfere with the production of granulocytes, and erythrocytes. Abdulkarimi (2011) reported that a single thyme administration did not affect the relative spleen weight. Giving mixed *Echinacea purpurea*, watercress, absinthe, and polygermander 10g / kg in broiler chicken feed had no effect on the spleen relative weight (Khaligh 2011). This indicates that the provision of plant material on the chicken does not affect the size of the spleen.

Bursa fabricius as the primary lymphoid organ with the humoral immune system plays a role in the synthesis and secretion of antibody substances into the blood circulation. In the combination of CAM, EM, and EDC the weight of the fabrication stock was not significantly different (P>0.05) with antibiotic treatment. The size of bursa fabricius affects the formation of antibodies, this indicates that the combination of CAM, EM, and EDC has a positive effect on antibody formation. Table 2 shows that although the relative weight of the bursa fabricius was not significant (P<0.05) between treatments, the combination of high and low powder produced a higher weight than antibiotic treatment. Likewise, Khaligh et al. (2011) reported that administration of a mixture of thyme extract 400 g, peppermint extract 300 g, green tea extract 200 g and licorice extract 100 g in broiler chickens showed the relative weight of the bursa fabricius there was no difference between both of them. Similarly, the administration of single thyme through drinking water does not show a difference with control (Abdulkarimi 2011).

Blood profile

The number of hematocrits, hemoglobin, lymphocytes, monocytes, and basophils were not significantly different (P>0.05) among all treatments (Table 2). This indicates that administration of a combination of CAM, EM, and EDC as a substitute for antibiotics do not interfere with the synthesis of hematocrit, hemoglobin, lymphocytes, monocytes, and basophils. Overall that antibiotic and bioactive combination treatments have no significant effect on the immune system. This can be seen from the size of the organ weight that does not affect the immune system. In fact, in vitro, the bioactive combination tested contained ingredients with a high antioxidant ability of *P. niruri* L, as reported by Sinurat et al. (2018). This is likely because the chickens that are raised are not challenged against diseases or bacteria.

Effect of the combination of CAM, EM, and EDC on total bacteria and number of *Escherichia coli* in the ileum

The combination of CAM, EM, and EDC in broiler chickens in the form of extracts or powder was not significantly different (P>0.05) against total bacteria and the number of E. coli in the ileum. However, with

Table 2. Effect of the combination of CAM, EM and EDC on life weight, the relative weight of spleen and bursa fabricius, and blood profile

Bioactive form and dosage	Life weight, g	Spleen weight	Bursa fabrisius weight	Hematocrit/ PVC (%)	Hemoglobin (g/dl)	Lymphocytes (%)	Monocytes (%)	Basophils (%)
		(% Life weight)						
Extract form:								
High dose	750.3[dc]	0.138	0.098	18.00	8.25	67.50	26.5	1.5
Medium dose	905.5[ab]	0.103	0.118	15.75	5.45	71.75	20	0
Low dose	806.8[bcd]	0.155	0.118	22.50	10.88	71.50	26.5	1.75
Powder form								
High dose	828.8[abc]	0.098	0.180	20.00	5.45	77.00	18.5	0.75
Medium dose	818.0[abc]	0.113	0.083	17.75	9.75	73.75	21.25	0.5
Low dose	927.8[ab]	0.085	0.195	23.75	10.50	69.75	30.25	0.5
Zn-Bacitracin	940.3[a]	0.103	0.163	16.00	4.00	60.50	14.5	0
Negative control	682.0[d]	0.073	0.090	20.25	10.45	74.25	18	0
Significance (P)	0.006	0.25	0.26	0.85	0.16	0.88	0.6395	0.6788

Description: Different subscripts in different lanes show significant differences (P <0.05)

the medium dose of extract treatment showed the total bacteria was lower than Zn-bacitracin treatment, this indicates that the combination of CAM, EM, and ED in medium dose can replace antibiotics. Likewise, the number of *E. coli* is still lower than the control which indicates that the combination treatment of CAM, EM, and ED can replace Zn-Bacitracin (Table 3).

The use of herb extracts as natural medicine has long been practised as an antimicrobial in the intestinal ecosystem to increase feed digestibility (Hernández et al. 2004). Attia et al. (2017) reported giving a mixture of oregano, fenugreek, chamomile and fennel extract reduced coliform bacteria and *E. coli*. The single administration of essential oil of *C. xanthorrhiza* or lemon peel did not affect the total bacteria in the ileum and cecum of broiler (Akbarian et al. 2013).

Effect of form and dose combination of CAM, EM, and EDC on total bacteria and number of *E. coli*

Treatment of combination of CAM, EM, EDC in broiler chickens between extract and powder form in high, medium, and low concentration was not significantly different (P>0.05) with Zn-Bacitracin treatment on total bacteria and the number of *E. coli* in the ileum (Table 4). This indicates that bioactive substances of three combinations (CAM, EM, EDC) have the same ability as Zn-Bacitracin as an antibacterial.

Himejima & Kubo (1991) reported that CAM had the ability to kill Gram-positive bacteria, where the bioactive compound of CNSL *Anacardium occidentale* L. was able to penetrate the membrane of bacterial lipid bilayers (Parasa et al. 2011), thus damaging its layer. The same thing happened to *E. coli* as described earlier that the defense of *E. coli* against the external environment decreases due to damage to cell walls by bioactive substances so that *E. coli* in the ileum cannot develop.

While the bioactive substances contained in *P. niruri* L) such as alkaloids saponins, tannins, phenols, glycosides can inhibit the growth of *E. coli* by damaging the membrane structure (Monte et al. 2014; Gbadamosi et al. 2015). Reportedly, *P. niruri* L extract at various concentrations can inhibit bacterial growth. The use of *P. niruri* L extract in vitro at a concentration

Table 3. Effect of the combination of CAM, EM, EDC on total bacteria and number of *E. coli in vivo*

Form and dose	Bacteria total (log cfu/ml)	*Escherichia coli* (log cfu/ml)
Extract:		
High dose	4.6775	2.677
Medium dose	3.3150	2.773
Low dose	4.9550	3.935
Powder:		
High dose	4.2350	2.657
Medium dose	4.2600	1.917
Low dose	5.2325	3.120
Zn-Bacitracin	4.8575	0.954
Negative control	4.9200	4.673

of 60% resulted in no visible growth of *S. dysenteriae* colonies (Munfaati et al. 2015), at the concentration of 10 mg/ml inhibited *E. coli* growth with inhibitory zones of up to 29 mm (Gbadamosi et al. 2015). *P. niruri* L. extract 0.0313 g/ml can inhibit the growth of *Edwardsiella tarda* bacteria (Sudarno et al. 2011), at concentrations of 1000 mg/mL also inhibit *E. coli*, *Staphylococcus aureus*, and *Salmonella typhi* (Ekwenye & Njoku 2006). *P. niruri* L. extract with a water fraction at a concentration of 0.75% can produce a 23 mm inhibition zone in *E. coli in vitro* (Lestariningsih et al. 2015).

Administration of Meniran (*P. niruri* L) extracts 1 ml/kg BW on broiler chicken that infected with *Mycoplasma gallisepticum* or CRD can improve the performance (Hidanah et al. 2017). As is known in previous *in vitro* studies that the treatment of the combination of CAM, EM, EDC extract can reduce the population of *E. coli*. The data above shows that administration of *P. niruri* L. plants in either extract or powder forms has the ability to eliminate *E. coli* populations *in vitro* and *in vivo* (in the digestive tract).

Eugenol as the most dominant bioactive substance in *Syzygium aromaticum* plants, with water extract at a concentration of 3% can inhibit the growth of *E. coli*, *Staphylococcus aureus* and *Bacillus cereus* (Cortés-Rojas et al. 2014). *S. aromaticum* extract with ethanol fraction had an 18 mm inhibition zone, with the methanol fraction having a 20 mm inhibition zone against *E. coli* (Pandey & Singh 2011). *S. aromaticum* extract at a concentration of 2000 ppm showed a 13 mm inhibition zone, 1500 ppm with a 9mm inhibition zone, and 1000 ppm with a 7 mm inhibition zone against *E. coli* (Kumar et al. 2014). The same thing can be seen in this study, that the lower the concentration of bioactive substances, the lower the inhibitory power.

The ability of each bioactive substance from the three plants, after being combined at moderate doses is the best for eliminating the population of *E. coli* in the chicken's digestive tract.

Effect of form and dosage of plant combinations on life weight, immune response, and blood profile.

Combination of CAM, EM, and EDC in broiler chickens in the form of extracts and powder in high, medium and low doses did not have a significant effect (P>0.05) on life weight, spleen weight, weight of the fabric, and blood profile, but real (P<0.05) against (Table 5). Statistically, the combination of CAM, EM, and ED does not affect the amount of hematocrit, production of macrophage and erythrocytes by spleen, does not affect the synthesis of antibodies by lymphocytes in the bursa fabicius. This illustrates that chickens are not lacked water, just as the process of transporting oxygen is not disturbed because hemoglobin synthesis is not disturbed. Monocytes that play a role in the immune system and basophil which is known to have the function of forming antibodies to react to allergies are also not affected by the combination treatment of CAM, EM, and EDC in broiler chickens.

Table 4. Effect of the form and dosage of the combination of CAM, EM, and EDC on total bacteria and the number of *E. coli* in the ileum

Form and concentration	Bacteria total (CFU/g)	*Escherichia coli* (CFU/g)
Form:		
Extract	4.7082	0.1327
Powder	4.5758	0.1180
Significance (P)	0.77	0.1135
Concentration:		
High	4.4563	0.1343 [a]
Medium	4.3286	0.1075 [b]
Low	5.0938	0.1371 [a]
Significance (P)	0.3309	0.0092
Form*Concentration	0.61	0.04

a, b In different lanes shows significant differences (P<0.05)

Table 5. Effect of forms and doses combination of CAM, EM, EDC on live weight, spleen, bursa fabricius, and blood profile

Form and concentration	Life weight (g)	Spleen (%)	Bursa Fabricius (%)	Hematocrit (%)	Hemoglobin (g/dl)	Lymphocytes (%)	Monocytes (%)	Basophils (%)
Form:								
Extract	820.8	3.13	0.15	22.50	10.92	70.3	29.20	4.33
Powder	858.2	2.57	0.17	22.36	10.28	73.5	28.00	1.75
Concentration:								
High	789.5	2.67	0.16	21.71	10.96	72.25	30.00	3.00
Medium	861.8	2.35	0.14	22.33	10.13	72.75	28.38	2.00
Low	867.3	3.53	0.18	23.13	10.69	70.63	27.50	3.00

CONCLUSION

The combination of cashew shell liquid of *Anacardium officinale* (CAM), *Phyllanthus niruri* L. (EM) extract, and clove leaf extract (EDC) can inhibit the growth of *Escherichia coli* and *Salmonella* sp. The best dose combination extract in inhibiting the growth of *E. coli* and *Salmonella* sp. is 100%, but the 25% dose has matched the AGP in vitro. In biological tests, a combination of CAM, EM, and EDC in the form of extracts with high, medium or low doses did not affect spleen weight, bursa fabricius, and blood profile. The best combination of CAM, EM, and EDC in the form of extracts or powder that reduces the total population of bacteria and the population of *E. coli* in the chicken intestine is a moderate dose. Likewise, for weight gain, there are indications that medium doses of extracts or powder can replace antibiotics. So it is concluded that the combination of CAM, EM, and EDC has the potential to replace AGP in poultry feed, especially on chicken.

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Blood Profile of Implantation Stainless Steel 316L Local Implant Material on Rat Femoral Bone

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ABSTRAK

Gustian, Soehartono H, Jujur N, Wargadipura AHS, Noviana D. 2018. Profil darah implantasi stainless steel 316L bahan implan lokal pada tulang femoralis tikus. JITV 23(3): 123-129. DOI: http://dx.doi.org/10.14334/jitv.v23i3.1836

Penelitian ini bertujuan untuk mengetahui profil darah tikus putih pasca-implantasi stainless steel 316L produk lokal asli Indonesia sebagai material implan metal tahan karat tidak terserap tubuh. Tiga puluh ekor ekor tikus strain sprague dawley (SD) jantan, dewasa, berusia 12 minggu dibagi ke dalam 3 kelompok yaitu kelompok kontrol tanpa implantasi, kelompok implan stainless steel 316L impor dan kelompok implan stainless steel 316L lokal yang dikembangkan Badan Pengkajian dan Penerapan Teknologi (BPPT). Pada kelompok perlakuan, material implan disisipkan diantara os femur dengan otot biceps femoris. Pada kelompok kontrol, os femur hanya dibuat defek tanpa dimasukkan material implan. Pemeriksaan respon sistemik dilakukan dengan pemeriksaan darah lengkap sebelum dan 30 hari sesudah implantasi. Hasil analisis pemeriksaan jumlah sel darah merah, kadar hemoglobin, nilai hematokrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), jumlah sel darah putih total dan diferensiasi sel darah putih antar kelompok perlakuan tidak menunjukkan perbedaan yang nyata. Sebagai kesimpulan, material implan stainless steel 316L impor maupun produk lokal tidak memberikan respon negatif terhadap profil darah hewan.

Kata Kunci: Stainless Steel, Implan Metal, Biokompatibilitas, Profil Darah, Reaksi Benda Asing

ABSTRACT

Gustian, Soehartono H, Jujur N, Wargadipura AHS, Noviana D. 2018. Blood profile of implantation stainless steel 316L local implant material on rat femoral bone. JITV 23(3): 123-129. DOI: http://dx.doi.org/10.14334/jitv.v23i3.1836

This study was aimed to obtain information regarding complete blood count (CBC) profile of post implantation of stainless steel (SS) 316L as an Indonesian local product of non-degradable metal implant. Thirty adult male rat, aged approximately 12 weeks were divided into 3 groups, i.e. control group without implantation, implant group with import SS316L and implant group with Indonesian national local SS316L that developed by Agency for the Assessment and Application of Technology (BPPT). The implant groups were given implants by inserting it between femoral bone and biceps femoris muscle. On the control group, defect was made on bone without inserting an implants material. Examination of the systemic response was done with CBC before and 30 days after implantation. The analysis of red blood cells amount, haemoglobin level, haematocrite value, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), total white blood cell and its differentiation from each group did not show significant differences. In conclusion, stainless steel 316L of import and national local product showed non-negative effects on blood profile.

Key Words: Stainless Steel, Metal Implant, Biocompatibility, Blood Profile, Foreign Body Reaction

INTRODUCTION

Nowadays, the technological advances in the field of materials for orthopaedic implantation have been highly developed (Nielsen et al. 2018). Bone is a major part of the body support, motion devices, body frame shaper and mineral storage (Kennedy et al. 2018). Various activities can be harmful to bones such as internal factors like age and external factors such as sports or even accidents (Lely et al. 2012). The bone damage can reduce its strength, thereby decreasing the mechanical functions of the body (McClung et al. 2018). Based on the results of research by the Research and Development Board of the Health Department Republic Indonesia in 2013, the number of injury incidents in Indonesia increased significantly from the year 2007. Common fracture cases occur in Indonesia caused by injuries such as falls, traffic accidents and sharp or blunt trauma (Kemenkes RI 2013). One way to deal with damage to bones and joints is to place biomaterials in the affected part (Krecisz et al. 2006).

Handling of fractures using metal pins such as stainless steel and titanium are mostly imported products (BPS 2012; Lely et al. 2012). Among the most common medical devices in Indonesia are imported products, while local health equipment products also require imported raw materials (PTM-BPPT 2017). Metallic biomaterials are often used to replace framework components of the human body such as artificial joints, bone plates, backbone attachments and tooth grafts (Bombac et al. 2007; Hafizi et al. 2016). The metallic biomaterial that is often used as an orthopaedic implant material is 316L stainless steel (SS316L) (Hafizi et al. 2016). Some studies consider that stainless steel has a corrosion resistance (Hermawan et al. 2011; Perren et al. 2017).

Some studies about local stainless steel casting manufacturers that use induction furnaces are highly dependent on imported raw materials (Jujur et al. 2015). The increasing demand of the SS316L orthopaedic material every year drives Indonesia to have a cheaper local substitute which availability can always be ensured (BPS 2012). The improvement of the infrastructure and an enormous amounts of natural resources as raw materials in Indonesia, reinforced the development of 316L stainless steel local products (Jujur et al. 2015). Stainless steel is an alloy of steel, iron, nickel and chromium containing the form a protective layer of oxidation, therefore this material is practically immune to rusting and corrosion (Lely et al. 2012; Manivasagam et al. 2010).

The Agency for the Assessment and Application of Technology (BPPT) together with local industry is developing the production of stainless steel implant components based on local resources for the medical equipment industry. The development is done to increase the added value of local resources in order to substitute the import of raw materials, health equipment products and to improve the independence of the nation. The components composition of stainless steel 316L local implant material are based on the import stainless steel 316L, in accordance with American Society for Testing and Materials (ASTM) number F-138. This material is the first material based from local raw and made in Indonesia (with government program) (Jujur et al. 2015). The local stainless-steel products are required to take a series of biocompatibility tests, in order to be used and legally distributed on the market in accordance with medical equipment standard (Hamidi et al. 2017). Blood tests as one of the biocompatibility tests will indicate a particular physiological condition of the individual (Keohane et al. 2015), therefore blood can be one of the main parameters in preclinical / biomedical research. This research needs to be done to find out animal blood profile on implantation of 316L stainless steel biomaterials of the local product as one of biocompatibility test (Krecisz et al. 2006; Larsson et al. 2007). It will indicate the body's systemic response to acceptance or rejection of implanted material (Fitria & Sarto 2014).

MATERIALS AND METHODS

Period and study place

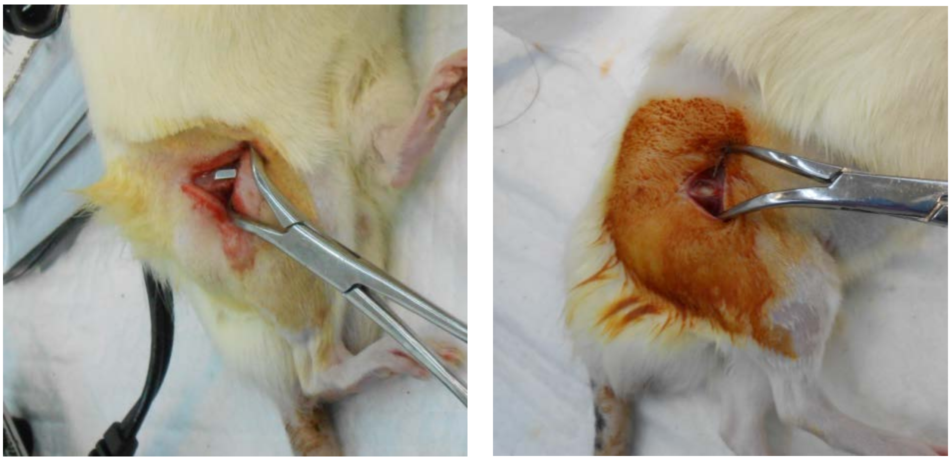
This research was conducted at the Veterinary Teaching Hospital Faculty of Veterinary Medicine of Bogor Agricultural University. This research was approved by Animal Ethics Committee Institute of Bogor Agricultural University with certificate number 79-2017 IPB.

Research material

Sprague Dawley rats aged 16 weeks with a range of 250-300 g weight were used in this study. 30 experimental rats derived from Indonesian Food and Drug Supervisory Agency were divided into three treatment groups: group without implantation as a negative control, 316L stainless steel implantation imported group as well as 316L stainless steel implantation local group which resulted from the cooperation and development of Agency for Assessment and Application of Technology. Ten rats in each group were divided randomly.

The equipment used were blood sample, mouse cage, syringe, rat restraint device, operating table, shaver, one unit of minor operation equipment including surgical shears, tissue forceps, haemostatic clamp, clamp towel, sterile tampon, scalpel, blade, needle holder, cotton, gauze, razor, needle and sewing threads absorbed by body polyglactin, dental drill, syringe, vacuum tube containing anticoagulant Ethylenediamine Tetraacetic Acid (EDTA) as blood samples container and blood test equipment.

The chemical composition requirements for stainless steel bone implants were specified in the ASTM F 138 (Laing 1979) and ISO 5832-1 specifications. The limits of the chemical composition for bone implants in both the ASTM and ISO standards were nearly identical. The limit were 0.03% Carbon, 0.75% siclicon, 2.00% for mangan, 0.025 for phosphate, 0.01% for sulphur, 17–19% chromium, 13–15% nickel, 2.25–3.0% molibdenum, 0.1% nitrogen, 0.5 cuprum and balance ferrum. The local stainless steel made by BPPT is based from that decision. The result of scanning electron microscope (SEM) that was equipped with EDS from this medical grade stainless steel 316L national local product were 0.028% Carbon, 0.610% siclicon, 1.20% for mangan, 0.025 for phosphate, 0.0039% for sulphur, 18.86% chromium, 10.89% nickel, 2.13% molibdenum, and balance ferrum (Jujur et al. 2015).



(A) (B)

Figure 1. Implantation area without material (A), material implanted on os femur defect (B).

Implantation of material

The 316L stainless steel implantable biomaterial of a local product developed by the Materials Technology Center of the Agency for Assessment and Application of Technology (PTM-BPPT) is 5x2x1 mm in size. The implantation in rats was performed by aseptic surgery. Prior to surgery, the surgical device was sterilized at an oven with a temperature of 121°C for 1 hour and the equipment carrier was sterilized at 75°C for 1 hour. The process of surgery begins with the preparation of animals and the preparation of operators in accordance with the procedure. All surgical procedures were under the influence of the Ketamine-Xylazine anaesthetic agent. Anaesthesia was performed intraperitoneally with a dose of 40 mg/kgBW and 5 mg/kgBW, respectively. After the anaesthesia process was completed and the animal in anaesthetic condition, hair was shaved in the lateral area of the right leg femoral rear and disinfected using 70% alcohol and 10% povidone-iodine.

Figure 1 shows the surgery performed on the lateral of the right leg os femur of the experimental animal. Skin and musculus biceps femoris is slashed along muscle fibre and parallel to the bone position so that the injured area does not dilate and facilitate the placement of implantable material. Muscles were exposed to the appearance of the os femur after the skin and muscles are slashed. The diafisis of os femur area was then scraped using dental drill to make bone defects. The erosion should be done carefully so that the penetration of the drill was not too deep and in accordance with the expected size. The part of the scraped bone is adjusted to the size of the implant material that is 5x2x1 mm. The implantable material was planted in accordance with its position and fixed to remain in the same place. On the control group, defect was made on bone without inserting an implants material, but the treatment was same with other groups. Closing the network was done by sewing a simple method using a special sewing thread. Post-operative treatment was administered with antibiotic amoxicillin and clavulanic acid orally at a dose of 15 mg/kg body weight 2 times daily for 7 days. After the implantation process is completed then done the observation periodically according to the test conducted.

Blood sampling

Blood sampling was performed in all aseptic treatment groups performed on day 7 prior to implantation and the 30th day after implantation. Blood sampling was done through animal tail using 3 ml syringe.

Firstly, rats were anesthetized by a combination of ketamine and xylazine anaesthesia with doses of 40 mg/kgBW and 5 mg/kgBW, respectively. The tail area is disinfected with alcohol and a blood sample was taken at least 1 ml inserted into a purple vacuum tube containing EDTA for haematological examination. Blood sample collected was temporarily stored in the cooling box until a laboratory examination.

The examination was done on complete blood count including the amounts of erythrocytes, leucocytes, platelets, haemoglobin, haematocrit, Mean Corpuscular Volume/MCV (Mean volume of one erythrocyte grain), Mean Corpuscular Haemoglobin/MCH (mean weight haemoglobin in one erythrocyte), Mean Corpuscular Haemoglobin Concentration (MCHC) (mean haemoglobin concentration in one erythrocyte) and differential leucocytes such as basophils, eosinophils, neutrophils, lymphocytes and monocytes. The direct blood image will show the systemic animal condition of the body's response to orthopaedic biomaterials implant.

Experiment design and data analysis

The experimental rats were divided into three treatments in a complete randomized design. Data were analysed using ANOVA. If treatment is significant, then the difference in mean value is tested by Duncan test (Steel & Torrie 1995).

RESULTS AND DISCUSSION

Red Blood Cell (RBC), hemoglobin, platelets, hematocrit, MCV, MCH, and MCHC

The description of the red blood cells, haemoglobin, thrombocytes, haematocrit, MCV, MCH and MCHC are presented in Table 1. The RBC shows no significant difference in the SS316L imported implant treatment group, local SS316L and control at day 7 before and day 30 after the implantation.

The haemoglobin (Hb) rate of rats in Table 1 showed no significant difference between the control group, the import implant treatment group and the local implant treatment group on day 7 before implantation and day 30 after the implantation.

The haematocrit values of the control group and the implant treatment group on day 7 before and day 30 after implantation showed no significant difference. The values obtained by examination on these three parameters are still included in the normal range of values according to the control group without implantation.

MCV, MCH and MCHC values before and after implantation in each treatment were not significantly different. MCV, MCH and MCHC values may indicate anaemia in animals (Sinno & Prakash 2013), but on the basis of blood tests obtained between implantable control treatments, implantation with imported SS316L and implantation with SS316L local products showed no significant difference. This is in line with the results of erythrocyte examination, which means that the implanted material provided has no effect on the systemic condition of the experimental animals.

Overview of white blood cells and differential leukocytes

Description of the white blood cell and differential leukocyte is presented in Table 2. The white blood cell in the control group, the SS316L imported implant treatment group and the local SS316L on day 7 before and day 30 after implantation showed no significant difference. The percentage of differential leukocytes such as basophils, eosinophils, banded netrophils, segment netrophils, lymphocytes and monocytes in the SS316L imported implant treatment group, local SS316L and control groups at day 7 before and 30th day after implantation showed no significant difference The percentage of white blood of rat and its differentiation include basophils, eosinophils, stem neutrophils, netrophil segments, lymphocytes and monocytes between controls and both implantation stainless steel 316L imported and local treatment did not decrease or increase significantly from seven days

Table 1. Red blood cell (RBC) profile, haemoglobin, platelets, haematocrit, MCV, MCH, and MCHC rats before and after 316L stainless steel implantation

Blood Parameters	H-7 Before Implantation			H+30 After Implantation		
	Control	Import SS316L	Local SS316L	Control	Import SS316L	Local SS316L
Hb	12.28±0.82	11.91±0.56	12.28±0.58	14.30±0.52	14.16±1.36	14.00±0.85
Platelet	279.69±49.56	278.82±51.52	302.08±66.22	401.62±36.83[b]	353.00±57.83[a]	332.67±56.63[a]
Ht	36.85±2.41	35.73±1.85	36.92±1.73	42.62±1.45	41.82±3.75	41.67±2.35
Eritrosit	4.06±0.26	3.95±0.19	4.05±0.21	4.74±0.17	4.70±0.45	4.65±0.29
MCV	89.85±0.38	90.00±0.00	89.67±0.49	88.31±0.63	88.45±0.69	88.67±0.65
MCH	30.00±0.00	30.00±0.00	30.00±0.00	30.00±0.00	30.00±0.00	30.00±0.00
MCHC	33.15±0.38	33.00±0.00	33.33±0.49	33.15±0.38	33.36±0.50	33.25±0.45

Description: Different notations on the same line show very significant differences (P <0.01)
Hb : Hemoglobin
Ht : Hematokrit
MCV : Mean Corpuscular Volume
MCH : Mean Corpuscular Haemoglobin
MCHC : Mean Corpuscular Haemoglobin Concentration

Table 2. White blood cell (WBC) and rat leukocyte differential profile before and after 316L stainless steel implantation

Blood Parameters	H-7 Before Implantation			H+30 After Implantation		
	Control	Import SS316L	Local SS316L	Control	Import SS316L	Local SS316L
Leukocyte	8.75±2.84	8.98±2.31	8.68±1.75	9.08 ±2.10	9.61 ±2.16	7.85±2.21
Basophils	0	0	0	0	0	0
Eusinophils	1.38±052	1.17±0.41	1.17±0.41	1.33±0.58	1.00±0.00	1.33±0.58
N. Banded	2.45±0.52	2.00±0.00	2.22±0.44	2.00±0.00	2.00±0.53	2.00±0.00
N.Segment	63.00±8.24	64.45±7.98	65.50±5.40	70.31±5.68	70.64±6.53	69.92±6.40
Limphocytes	30.23±5.90	29.64±6.41	28.58 4.29	25.31±4.35	24.27±6.05	25.92±6.16
Monocytes	3.85±1.63	2.91±1.38	3.67±1.67	2.92±1.44	3.73±1.27	2.50±0.80

Description : Different notations on the same line show very significant differences (P <0.01)
N. Banded : Banded Netrophils
N. Segment : Segmented Netrophils

before and thirty days after implantation. These results suggest that local SS316L and imported SS316L implants have no effect on the white blood cell profile and the systemic white blood cell differential.

Discussion

Red Blood Cell (RBC), hemoglobin, platelets, hematocrit, MCV, MCH, and MCHC

In this study, rat's body does not have a deficiency of red blood cells when the implantation process is done and it can be assumed that the implantation process works well (Perren et al. 2017). Haemolysis may occur as a result of a foreign material inserted into the body causing rupture of red blood cells, it will also be directly related to the amount of haemoglobin in the body that would indicate the acceptance or rejection of the foreign body in the body (Keohane et al. 2015). In this case, the results obtained between both erythrocytes and haemoglobin were not significantly different between treatments which means that there was no effect on the body on the implantation of the material.

Thrombocyte levels in the control group without implantation increased on the thirtieth day after implantation compared to the treatment group with imported and local SS316L implantation. The main function of thrombocyte is to protect blood vessels against endothelial damage due to trauma as a prelude

o healing wounds in the walls of blood vessels. The implantation procedure in this study through the operation process can cause injury, damage to skin tissue and muscle damage due to slicing, while damage to bone also occurs due to the manufacture of defects. The tissue damage leads to wound healing reactions that begin with acute inflammation and blood clots by platelets (Perren et al. 2017).

In the event of injury, the damaged tissue thrombocyte will release thromboplastin which reacts with the protrombin and calcium to form thrombin. The thrombin will react with fibrinogen to form fibrin which will cover the wounded tissue (Fitria & Sarto 2014; Sinno & Prakash 2013). The decline in thrombocyte count in the blood circulation at the onset of the injury will accompany the event. Levels of thrombocyte on negative control after implantation experienced a significantly different increase slammed two other treatment groups, this can be due to the surgical process cut wounds and bone made defects without implantation of the material, resulting in wound tissue more than two other treatments. As early as the process of wound, thrombocyte in the circulation decreases as most of the thrombocyte lead to injured tissue in the surgical area (Anderson et al. 2008). Thirty days after implantation, the body has produced large amounts of thrombocyte to suffer the number of thrombocyte that continue to be sent to the tissues, thereby still finding high thrombocyte counts in the circulation (Sinno & Prakash 2013). This is also in accordance with Keohane's et al. (2015) statement, the production of thrombocyte by the body will continue to be done as a reciprocal of the decline in the number of thrombocyte in the circulation. The level of thrombocyte in this case was in normal level in accordance to the control group without implantation.

Overview of white blood cells and differential leukocytes

The first tissue in the body that will always be the entrance for foreign bodies including the implant material was blood, it goes through a series of bodily biological processes followed by various bodily responses (Anderson 2001; Keohane et al. 2015). The implant planting operation process in this study will also affect the profile of white blood cells from animals. The body's response processes that generally follow the process were haemolysis, blood clot formation and then inflammatory processes that will involve white blood cells (Perren et al. 2017). Implantation of biomaterials in the animal body was a form of injury, a process of interaction between blood and material, acute and chronic inflammation, granulation tissue formation and fibrous capsule formation or fibrosis (Anderson et al. 2008). Components of white blood cells such as basophils, eosinophils, banded neutrophils, segmented neutrophils, lymphocytes and monocytes are small in normal conditions, but when there are antigens or foreign bodies such as implantable material inserted into the body, the body reacts, resulting in an increase in the blood cell component (Keohane et al. 2015).

White blood cells serve as the body's defense system against infectious agents as well as foreign objects that enter the body (Saputri et al. 2012). White blood cells are formed in the bone marrow and lymphoid tissues which are then circulated to all parts of the body that are in need especially during infectious and inflammatory processes (Keohane et al. 2015).

In this study, the percentage of white blood of rat and its differentiation include basophils, eosinophils, stem neutrophils, netrophil segments, lymphocytes and monocytes between controls and both implantation stainless steel 316L imported and local treatment did not decrease or increase significantly. These results suggest that local SS316L and imported SS316L implants have no effect on the white blood cell profile and the systemic white blood cell differential (Keohane et al. 2015; Saputri et al. 2012).

The results obtained on monocyte calculations was increase that occurred 30 days after implantation, but the figures obtained did not show a real difference. This is in accordance with Anderson (2001) statement, on the inflammatory process due to implantation of foreign matter, the first few days will be dominated by neutrophils which are then replaced by monocytes. The subsequent monocytes differentiate into macrophages (Anderson 2001).

CONCLUSION

In this researh, we investigated the medical grade 316L stainless steel biocompatibility from blood profile. The result of this study shows that material implant of import and medical grade 316L stainless steel of local product are not different significantly with control (without implantation), stainless steel 316L of import and national local product showed non-negative effects on blood profile.

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Ultrasonographic and Vaginal Cytological Diagnostics of the Queen

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ABSTRAK

Pertiwi AP, Tumbelaka LITA, Ulum MF. 2018. Ultrasonografi dan diagnosa sitologi vagina dari kucing betina. JITV 23(3): 130-142. DOI: http://dx.doi.org/10.14334/jitv.v23i3.1809

Ultrasonografi adalah metode diagnosis untuk mencitrakan struktur organ reproduksi, dan hal ini harus didukung oleh sitologi vagina untuk mengidentifikasi aktivitas ovarium berdasarkan jenis sel epitel vagina. Tujuan dari penelitian ini adalah untuk mengobservasi tampilan organ reproduksi kucing betina yang didukung dengan pemeriksaan sitologi vagina. Kucing betina sebanyak 10 ekor digunakan dalam penelitian ini yang dikelompokkan berdasarkan anamnesis dan sejarah medis sebagai kelompok intact (n=5) dan yang telah diovariohisterektomi (OH) (n=5). Vagina, serviks, korpus dan kornua uterus, serta ovarium dicitrakan dan diukur menggunakan ultrasonografi. Vagina, korpus dan kornua uterus tampak sebagai struktur seperti pipa dengan garis luar yang hiperekoik. Lumen pada korpus dan kornua uterus terlihat hiperekoik. Ovarium terlihat sebagai struktur berbentuk bulat atau oval dengan folikel yang anekoik. Korpus luteum berdinding tebal dan terlihat anekoik pada bagian pusatnya. Korpus albikan terlihat sebagai struktur hiperekoik. Vagina kucing betina OH terlihat berukuran lebih pendek daripada kucing betina intact. Serviks terlihat sebagai struktur hiperekoik yang menghubungkan vagina dan korpus uterus. Jenis-jenis sel epitel vagina juga diidentifikasi dan dihitung. Hasil sitologi vagina menunjukkan beberapa status aktivitas yang berbeda pada ovarium. Proestrus teridentifikasi pada 3 kucing intact, metestrus akhir pada 2 kucing intact dan 4 kucing OH, serta anestrus pada 1 kucing OH. Morfologi uterus dan servik juga dipengaruhi oleh aktifitas ovarium.

Kata Kunci: Status Estrus, Kucing Betina, Organ Reproduksi, Ultrasonografi, Sitologi Vagina

ABSTRACT

Pertiwi AP, Tumbelaka LITA, Ulum MF. 2018 Ultrasonographic and vaginal cytological diagnostics of the Queen. JITV 23(3): 130-142. DOI: http://dx.doi.org/10.14334/jitv.v23i3.1809

Ultrasonography is a diagnostic method to image the conditions of reproductive organs and it could be supported by vaginal cytology to identify the activities of the ovaries by the types of vaginal exfoliate cells. The aims of this study was to observe reproduction organ through ultrasonography with supportive diagnostic with vaginal cytological assessment. A total of 10 individual queens were used in this study and then grouped into intact group (n=5) and spayed (ovariohysterectomy) group (n=5) based on the anamneses or their medical history. The vagina, cervix, uterus body and horns, and ovaries were imaged and measured by ultrasound. Vagina, uterine body and horn seem as pipe-like structures with hyperechoic outer lines. The lumen in uterine body and horn seem as a hyperechoic structure. The ovaries seem as round- or oval-shaped structures with anechoic follicles. The corpus luteal has thick wall and seen as anechoic in its centre part. The corpus albicans seems as a hyperechoic structure. The vagina of spayed queens seemed more corrugated than those intact queens. The cervix is seen as a hyperechoic structure linking the vagina and uterine body. Exfoliate vaginal epithelial cell types were then also be identified and counted on each queens. The results of vaginal cytology showed that proestrus occured in 3 intact queens, late metestrus in 1 intact and 3 spayed queens, anestrus in 1 spayed queen, and unidentifiable estrus stage in 1 intact and 1 spayed queens. Moreover, the morphology of cervix and uterine was affected by the activity of ovary.

Key Words: Estrus State, Queen, Reproductive Organs, Ultrasonography, Vaginal Cytology

INTRODUCTION

The importance of reproductive organ ultrasonography in diagnosing estrus state in queen such as estrus or estrus disorder, is well established and documented (Davidson & Baker 2009; Malandain et al. 2011). The indicators evaluated are size of uterine wall tissue and size of ovary including follicles, corpus luteum, and other ovarian tissues in an intact queen. However, spayed queens in several case has been reported shows a sign of estrus that in ultrasonography examination was not find the ovary. This condition was knows as ovary remnant syndrome (ORS) (Wallace 1991) that cannot independently assessed by vaginal cytology (Mills et al. 1979). Even though the diagnostic of ultrasonography cannot image the systemic status of reproductive status, it is valuable diagnostic tool to image internal architecture of reproductive organ tissue, locally. Ultrasound imaging nowadays is used to assess the reproductive organ in several species in Indonesia, such as cat and dog (Noviana et al. 2012), kacang goat (Santoso et al. 2014), garut sheep (Amrozi & Setiawan 2011; Gunawan et al. 2012), horse (Rahman 2012), also timor deer (Prawigit 2007), tom (Ulum et al. 2017), and phyton (Lestari et al. 2017).

Another method used in order to assess reproductive (estrus) status in systemic state indirectly, vaginal cytology, also well established and documented (Mattos et al. 2003; Mills et al. 1979). In this vaginal cytology examination, the presence of exfoliate vaginal cells was represented the systemic status of reproductive organ activity. This method can determine the stage of oestrus cycle, endocrine and reproduction pathology indirectly in animals (Mattos et al. 2003). This is caused by the high responsivity of vaginal epithelial cells towards hormone fluctuation, especially estrogen which will causing changes of vaginal cellular profiles (Erünal-Maral et al. 2000). The use of vaginal cytology in determining the stage of oestrous cycle is well applied in dog (Reddy et al. 2011), Ettawa grade goat (Satria et al. 2016), and javan mongoose (Zora 2014).

Although the two methods were used individually in veterinary clinical services, however, no prospective study of the subject has yet been conducted, and regard to these two methods in their ability to diagnose reproductive status in precisely. The aims of this study were to assess the mutual support between ultrasonography findings with vaginal cytological results, and to establish a logical approach concerning the presence of estrus cycle in queens. In this study, totally ten of intact and spayed queens were undergo gynecological examination by ultrasonography scanning and vaginal cytology. Sonogram of reproductive organs measurements and exfoliate vaginal cell count were compared in order to diagnose the estrus state of each queens.

MATERIALS AND METHODS

Animals

Two groups of queens used in this study were the intact queens (n=5) and the spayed queens (n=5). The cats were obtained from the inside and surrounding area of Bogor Agricultural University, some of them were strays and the others were owned as pet animals. The queen's age was determined by examining its body and teeth condition. Age determination was done based on the criteria made by The Humane Society of the United States (1996): at 5–7 months old, all teeth have changed into permanent teeth; at 1–2 years old, the teeth may appear dull with some tartar build-up on back teeth; at 3–5 years old, the teeth show more tartar build-up on all teeth and some tooth wear; at 10–15 years old, the teeth are worn and show heavy tartar build-up, and some teeth may be missing. Nulliparous and multiparous status were determined based on observation of the abdomen and nipple condition. Multiparous individuals have more protractile nipples than nulliparous individuals (WHO & UNICEF 2009).

Ultrasound imaging

The queens were physically restrained without anesthesia or sedation. Abdomen hair was also not trimmed for ultrasound. Ultrasound gel (Hamco, PT Bintang Putra Pratama, Indonesia) was applied on the hypogastric area of the abdomen. Sonogram was taken using an ultrasound console (SonoDop S-3X, PT Karindo Alkestron Indonesia) with a linear transducer owing 10 MHz of frequency in longitudinal and transversal views. The queen was positioned in dorsal recumbence and the sonogram was taken from the hypogastric part of the queen's ventral body (Figure 1).

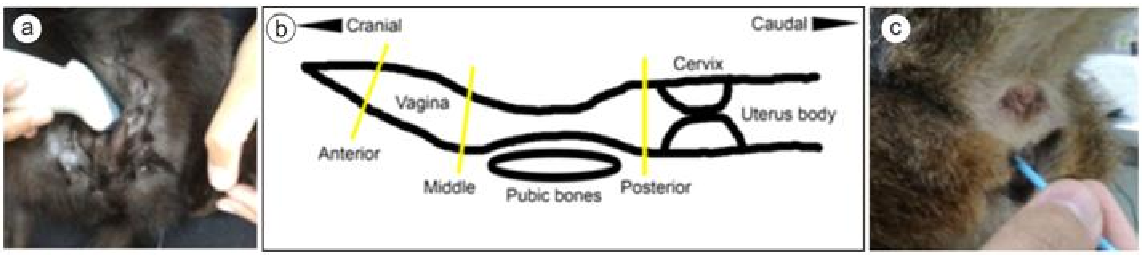

Figure 1. Ultrasonography imaging of reproductive organ and its vaginal swabbing of the queens.
The position of the transducer positioning for scanning of reproductive organ (A)
Illustration of the vaginal measurement parts (B)
The vaginal swabbing process (C).

Measurement the size of reproductive organ was done by ImageJ (NIH, USA) after the sonograms were saved in JPG format. Reproductive organs that assessed in sonogram of this study i.e. vagina, cervix, uterus body and horns, ovaries, corpus luteum, corpus albican, corpus rubrum, and follicles. Measurements size and number of each parts of reproductive organs were done in three repetitions. The measurements of vaginal size were done in three different spots i.e. anterior, middle, and posterior.

Vaginal cytology

The vagina of queens was the swabbed by using a cotton swab (Baby Huki, PT Ikapharmindo Putramas, Indonesia) that has been moistened with distilled water (Figure 1). The tip of the cotton swab was put into the vulva, slightly inclinated and then pushed inside gently. Swab was done by rotating the tip clockwise 2–3 times. The tip was then smeared onto the surface of an object glass and left to dry. The vaginal smear was then fixated using methanol for 5 minutes and stained with 10% of Giemsa (Merck, PT Merck Tbk, Indonesia) for 30 minutes. The stained results were observed with light microscope (Olympus CX31, PT Fajar Mas Murni, Indonesia) using 4x, 10x, and 40x of objective magnifications. Photos of microscope observation were taken using a digital microscope camera (HDMI & USB Multioutput HD Camera Indomicro, Indonesia). The proportion of parabasal, intermediate, and superficial vaginal epithelial cells per 100 epithelial cells and neutrophils per 2 fields of view were counted to determine the stage of the estrus. Vaginal epithelial cell type determination was based on (Cowell et al. 2007; Mills et al. 1979) which categorized the cells as below: superficial epithelial cell is the biggest type with small nucleus (oval-shaped and will become picnotic and disappear later); intermediate epithelial cell is approximately two times larger than the parabasal epithelial cell, it is round- to oval-shaped with a vesicular to flattened nucleus; parabasal epithelial cell is the smallest type, with almost-similar sizes and shapes, a big vesicular nucleus and a little cytoplasm. The stage of the estrus determination was based on (Mills et al. 1979).

Data analysis

Obtained data of sonogram and its measurement were presented and descripted narratively. Exfoliate vaginal cell counting from vaginal cytology were analyzed and presented to show the estrus stage of each queens.

RESULTS AND DISCUSSION

Table 1. shows the profile of all of the queens that used in this study, which consisted of 8 Domestic cats and 2 Persian-mix cats. All queens did not show behavioral signs of estrous when the ultrasound was being done. Queens Intact 1 and 5 were multiparous, while queens Intact 2, 3, and 4 were nulliparous.

Table 1. Profile of the queens that used in the study

Queen ID	Race	Status (Stray/Pet)	Age (months)	Weight (kg)	Estrus Behavior	Nulliparous/Multiparous
Intact 1	Domestic	Stray	20	3.0	None	Multiparous
Intact 2	Domestic	Pet	14	2.0	None	Nulliparous
Intact 3	Domestic	Pet	12	1.9	None	Nulliparous
Intact 4	Domestic	Stray	5	2.0	None	Nulliparous
Intact 5	Domestic	Stray	14	3.2	None	Multiparous
Spayed 1	Domestic	Stray	24	3.6	None	n.a.
Spayed 2	Domestic	Pet	8	4.2	None	n.a.
Spayed 3	Persian mix	Pet	84	3.5	None	n.a.
Spayed 4	Persian mix	Pet	47	3.7	None	n.a.
Spayed 5	Domestic	Stray	80	2.8	None	n.a.
Intact = 5, Spayed =5	Domestic=8; Persian mix=2	Pet=5; Stray=5	8-84	1.9-4.2	None	Nulliparous=3; Multiparous=2

Note: n.a.=not available, where the nulliparous/multiparous status was not determined in spayed queens

Sonogram of vagina and cervix

Sonogram of vagina was seen as an anechoic pipe-like structure with hyperechoic walls that narrowed caudally (Figure 2). Table 2 shows the measurement results of vagina and cervix. Measurement of spayed queen vagina was done only at the front and the middle parts of the vagina. The average diameter of the front part of intact queen's vagina (2.58±0.26 mm) was smaller than the spayed ones (4.02±0.64 mm).

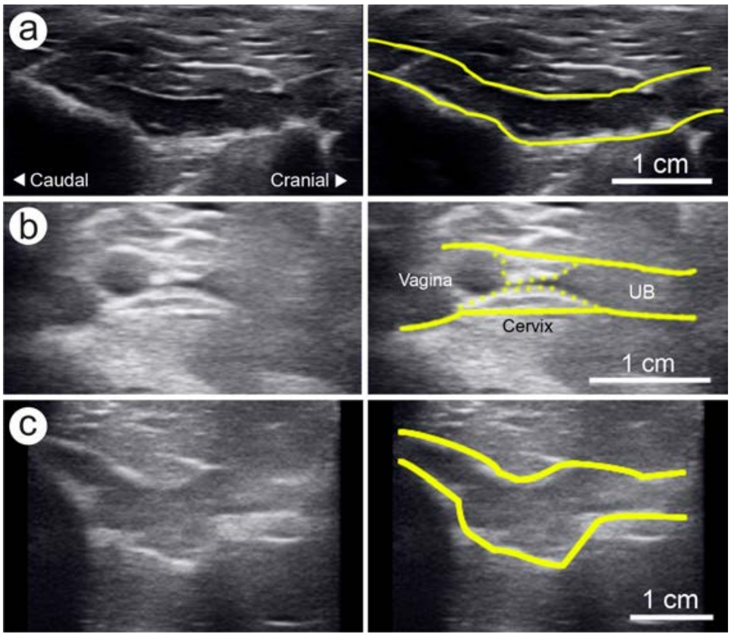

Figure 2. Sonograms of vagina, cervix, and uterine body of the queens. Sonograms of vagina queen Intact 1 **(a)**, vagina of queen Spayed 1 **(b)**, and vagina, cervix, and uterus body (UB) of queen Intact 1 **(c)**. Cervix (interrupted lines) seems hyperechoic, and located between the vagina and the uterus body.

Table 2. Size of vagina and cervix of the queens

Queen ID	Vagina			Cervix			
	Diameter of anterior (mm)	Diameter of middle (mm)	Diameter of posterior (mm)	Length (mm)	Diameter (mm)	Thickness of ventral layer (mm)	Thickness of dorsal layer (mm)
Intact 1	2.96±0.56	4.48±0.29	3.79±0.07	5.36±0.37	4.14±0.04	1.88±0.35	1.74±0.25
Intact 2	2.32±0.37	3.63±0.09	2.33±0.06	5.81±0.49	2.58±0.11	1.15±0.27	1.05±0.12
Intact 3	2.71±0.18	4.01±0.33	3.00±0.15	3.75±0.29	2.77±0.10	0.63±0.12	1.23±0.15
Intact 4	2.40±0.25	3.20±0.21	3.73±0.32	5.83±0.11	2.93±0.08	0.63±0.19	1.75±0.17
Intact 5	2.50±0.44	4.80±0.13	6.36±0.15	10.22±0.54	5.89±0.17	2.65±0.12	2.36±0.08
Mean intact	2.58±0.26	4.02±0.64	3.84±1.53	6.19±2.41	3.66±1.39	1.39±0.87	1.63±0.51
Spayed 1	3.09±0.38	4.70±0.35	-	-	-	-	-
Spayed 2	3.32±0.24	3.81±0.33	-	-	-	-	-
Spayed 3	3.76±0.12	5.74±0.72	-	-	-	-	-
Spayed 4	4.04±0.28	4.36±0.21	-	-	-	-	-
Spayed 5	3.01±0.39	4.74±0.18	-	-	-	-	-
Mean spayed	3.44±0.44	4.67±0.70	-	-	-	-	-

The average diameter of the middle part of intact queen's vagina (4.02±0.64 mm) was also smaller than the spayed ones (4.67±0.70 mm), but the front part of intact queen's vagina (2.58±0.26 mm) was smaller than the spayed ones (3.44±0.44 mm). The average diameter of the back part of spayed queen's vagina was 3.84±1.53 mm. The spayed queens' vagina was seemed shorter, while the intact queens' are straighter to the cranial (Figure 2). The average diameter of the five intact queens' cervix was 3.66±1.39 mm (Table 2). The cervix diameter of queens Intact 1 and 5 were larger than queens Intact 2, 3, and 4. The largest cervix was showed at queen Intact 5. The cervix is located between the vagina and the uterus and it's seemed as a hyperechoic structure with anechoic lumen (Figure 2).

Sonogram of uterine body and horns

Sonogram in Figure 3 shows the appearance of the uterine horns with lumen and without lumen. Uterus seems as an anechoic pipe-like structure with anechoic walls and lumen. The uterus of queen Intact 5 seems thicker than other intact queens. Uterus's outer layer (serosa) and lumen seem hyperechoic. The lumen of the uterus body was only seen in queen Intact 2, 3, and 4. The lumen of the uterus body was seen in all the intact queens. The average diameter of all the intact queens' uterus bodies and horns are 4.67±1.82 mm and 3.76±1.27 mm (Table 3).

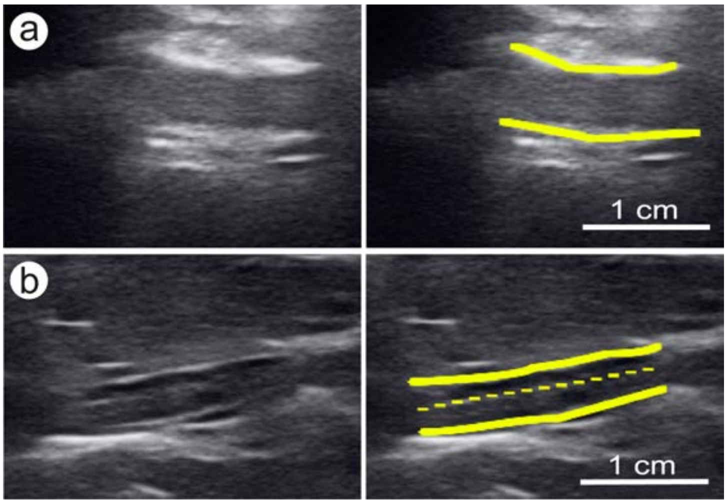

Figure 3. Sonograms of the uterine horn of the queens. The uterine horn without lumen **(a)** of queen Intact 1 and the uterine horn with lumen **(b)** of queen Intact 3. The lumen is indicated with interrupted line.

Table 3. Size of the uterus body and horn of the intact queens

Queen ID	Uterus body			Uterus horn		
	Diameter (mm)	Thickness of ventral layer (mm)	Thickness of dorsal layer (mm)	Diameter (mm)	Thickness of ventral layer (mm)	Thickness of dorsal layer (mm)
Intact 1	3.99±0.16	n.a.	n.a.	3.69±0.27	1.54±0.31	1.71±0.04
Intact 2	3.30±0.39	1.25±0.07	1.92±0.11	2.61±0.01	0.80±0.05	1.74±0.10
Intact 3	2.84±0.08	0.70±0.09	1.61±0.21	3.07±0.31	0.95±0.14	1.63±0.02
Intact 4	6.77±0.23	2.65±0.14	3.07±0.06	3.53±0.11	1.48±0.08	1.18±0.13
Intact 5	6.44±0.34	n.a.	n.a.	5.92±0.21	2.58±0.08	2.80±0.00
Mean intact	4.67±1.82	1.53±1.01	2.20±0.77	3.76±1.27	1.47±0.70	1.81±0.60

Note: n.a.= not available, not seen in sonogram clearly

Sonogram ovary

Figure 4 shows the sonogram of intact queen's ovary. The shape of ovary's body is oval or round and seen as hypoechoic structure. The size of ovary, follicles, and its corpora body were measured and shown in Table 4. The largest ovary was the right ovary of queen Intact 4 whose diameter was 17.32 mm, while the smallest one is the right ovary of queen Intact 2 whose diameter was 9.17 mm. Sonogram of follicles seem as anechoic, corpus luteum seems hypoechoic, corpus albicans seem hyperechoic, and corpus rubrum seem as round structure with anechoic centre. The follicles were varied in size, from the largest one with 5.82 mm diameter in the right ovary of queen Intact 5 to the smallest one with 2.21 mm diameter in the right ovary of queen Intact 1. Queen Intact 1 had the highest total of follicles, with 5 follicles: 3 in the right ovary and 2 in the left ovary. The highest total of luteal body was found in queen Intact 1 owing 6 corpus luteum. Queen Intact 4 had a corpus albicans in the right ovary and a corpus rubrum in the left ovary.

Vaginal cytology

The shape of vaginal epithelial cells and neutrophils can be seen in Figure 5 and then the results of vaginal smear cell counting were shows at Table 5. The count of vaginal cytology at queen Intact 1 shows that the almost-similar amounts of parabasal and intermediate epithelial cells, with a moderate amount of superficial epithelial cells. The vaginal cytology count of queens Intact 2 and Spayed 2, 3, 5 show the almost similar amounts of parabasal and intermediate epithelial cells, with a few of superficial epithelial cells. The vaginal cytology count of queen Intact 3, 4, and 5 is dominated by the amount of the intermediate epithelial cells, with almost-similar amounts of parabasal and superficial epithelial cells. The amount of intermediate cells is really dominant in vaginal smear of queen Spayed 4. The highest amount of neutrophils was found in queen Intact 1. A metestrum cell was found in vaginal smear of queen Intact 4 (Figure 5d).

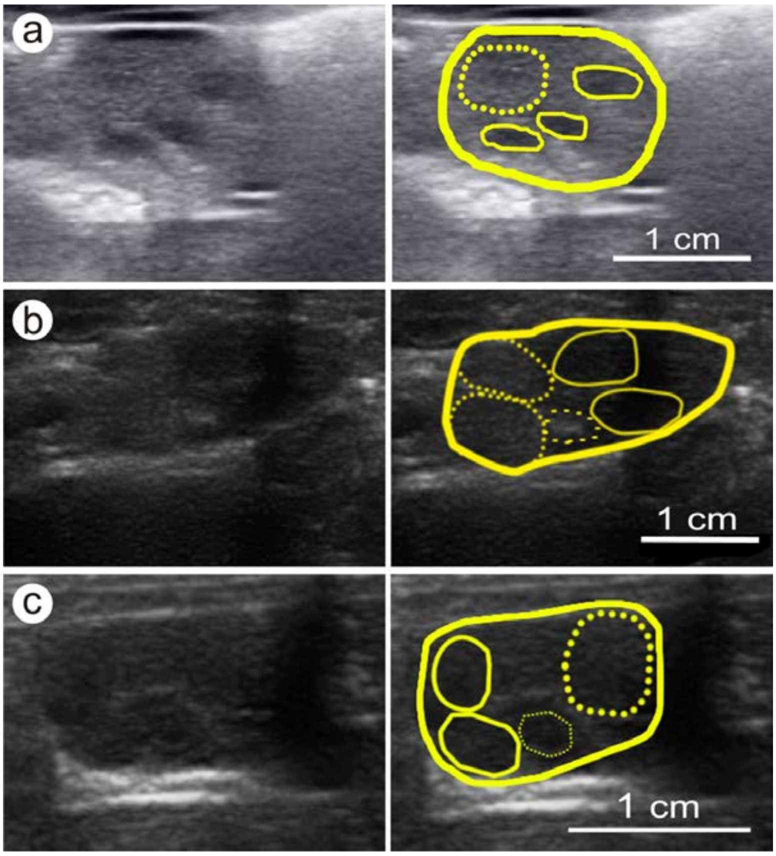

Figure 4. Sonograms of ovaries of the intact queens. The right ovary of intact 1's queen **(a)** with follicles (thin line) and CL (interrupted thick line); The right ovary of intact 4's queen **(b)** with follicles and corpus albican (far interrupted line); The ovary of intact 4's queen **(c)** with follicles, corpus luteum, and corpus rubrum (interrupted thin line).

Table 4. Diameter size and number of ovaries, follicles, corpus luteums, corpus albicans, and corpus rubrums on the intact queens

Parts of ovary	Queen ID					Mean intact
	Intact 1	Intact 2	Intact 3	Intact 4	Intact 5	
Diameter (mm); Right ovary						
Ovary's body	12.67	9.17	12.34	17.32	12.03	12.71±2.93
Follicle 1	2.35	-	-	4.13	5.82	
Follicle 2	2.21	-	-	3.96	3.41	3.52±1.26
Follicle 3	2.73	-	-	-	-	
Corpus luteum 1	5.90	4.13	5.05	5.79	3.93	4.83±0.88
Corpus luteum 2	-	-	5.04	5.28	3.51	
Corpus albican	-	-	-	3.05	-	3.05±0.00
Corpus rubrum	-	-	-	-	-	n.a.
Diameter (mm); Left ovary						
Ovary's body	12.49	10.09	12.33	11.33	11.69	11.59±0.96
Follicle 1	3.03	-	-	3.38	4.00	3.40±0.53
Follicle 2	4.09	-	-	3.07	2.85	
Corpus luteum 1	3.12	3.64	5.04	4.36	4.38	
Corpus luteum 2	3.51	-	-	-	4.19	
Corpus luteum 3	2.67	-	-	-	-	2.95±1.02
Corpus luteum 4	1.47	-	-	-	-	
Corpus luteum 5	2.89	-	-	-	-	
Corpus albican	-	-	-	-	-	n.a.
Corpus rubrum	-	-	-	2.44	-	2.44±0.00
Range diameter (mm); Right and left ovary						
Ovary's body	12.49-12.67	9.17-10.09	12.33-12.34	11.33-17.32	11.69-12.03	
Follicle	2.21-4.09	-	-	3.07-4.13	2.85-5.82	
Corpus luteum	1.47-5.90	3.64-4.13	5.04-5.05	4.36-5.79	3.51-4.38	
Corpus albican	-	-	-	3.05	-	
Corpus rubrum	-	-	-	2.44	-	
Number of ovary's parts; right ovary						
Follicle	3	-	-	2	2	2.33±0.58
Corpus luteum	1	1	2	2	2	1.60±0.55
Corpus albican	-	-	-	1	-	1.00±0.00
Corpus rubrum	-	-	-	-	-	n.a.
Number of ovary's parts; left ovary						
Follicle	2	-	-	2	2	2.00±0.00
Corpus luteum	5	1	1	1	2	2.00±1.73
Corpus albican	-	-	-	-	-	n.a.
Corpus rubrum	-	-	-	1	-	1.00±0.00
Total number of ovary's parts; right and left ovary						
Follicle	5	-	-	4	4	4.33±0.58
Corpus luteum	6	2	3	3	4	3.60±1.52
Corpus albican	-	-	-	1	-	1.00±0.00
Corpus rubrum	-	-	-	1	-	1.00±0.00

n.a.= not available

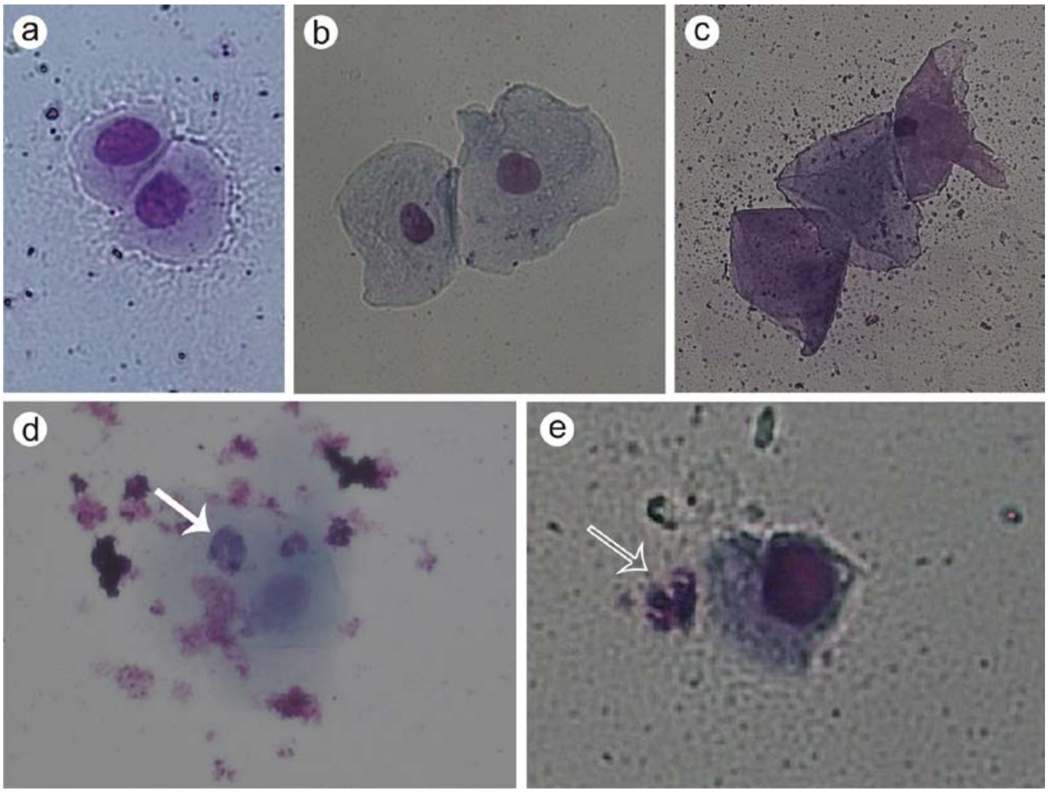

Figure 5. Cells found in vaginal smears. Parabasal **(a)**, intermediate **(b)**, nucleated and un-nucleated superficial **(c)** vaginal epithelial cells, metestrum cell (white arrow) and neutrophil (empty arrow) **(d)** seen from the vaginal smears of the queens that used in this study.

Table 5. Number of the queen's vaginal epithelial cell type

Queen ID	Vaginal epithelial cell type			Metestrum	Neutrophil
	Parabasal	Intermediate	Superficial		
Intact 1	41	44	15	0	4
Intact 2	61	37	2	0	0
Intact 3	18	53	29	0	2
Intact 4	25	57	18	4	1
Intact 5	14	59	27	0	0
Mean intact	31.8±19.3	50±9.3	18.2±10.8	0.8±1.8	1.4±1.7
Spayed 1	33	40	27	0	0
Spayed 2	66	30	4	0	3
Spayed 3	42	51	7	0	2
Spayed 4	8	75	17	0	3
Spayed 5	67	32	1	0	3
Mean spayed	43.2±24.7	45.6±18.4	11.2±10.7	0	2.2±1.3

n.s.=not specific (suspect hormone-insufficiency)

Diagnoses of estrus state based on vaginal cytology and ultrasonography

Figure 6 shows the percentage of exfoliate vaginal cells from the totally 10 queens that consist of 5 Intact and 5 Spayed queens. Based on the results showed that 4 of 10 queens diagnosed in metestrus, 3 of 10 in proestrus, 2 of 10 cannot defined the estrus state, and 1 queen diagnoses in anestrus or diestrus (Figure 6a). Further, the diameter of vagina and cervix of all queens has no different between the statuses of estrus (Figure 6b).

Figure 7 shows the diameter of uterus and ovary activity on the sonogram with its relation to estrus status based on vaginal cytology on the 5 intact queens. The uterus body diameter was higher (Figure 7a) on the queens that having ovary with follicles compared to ovary without follicles, only having corpus luteum (Figure 7b).

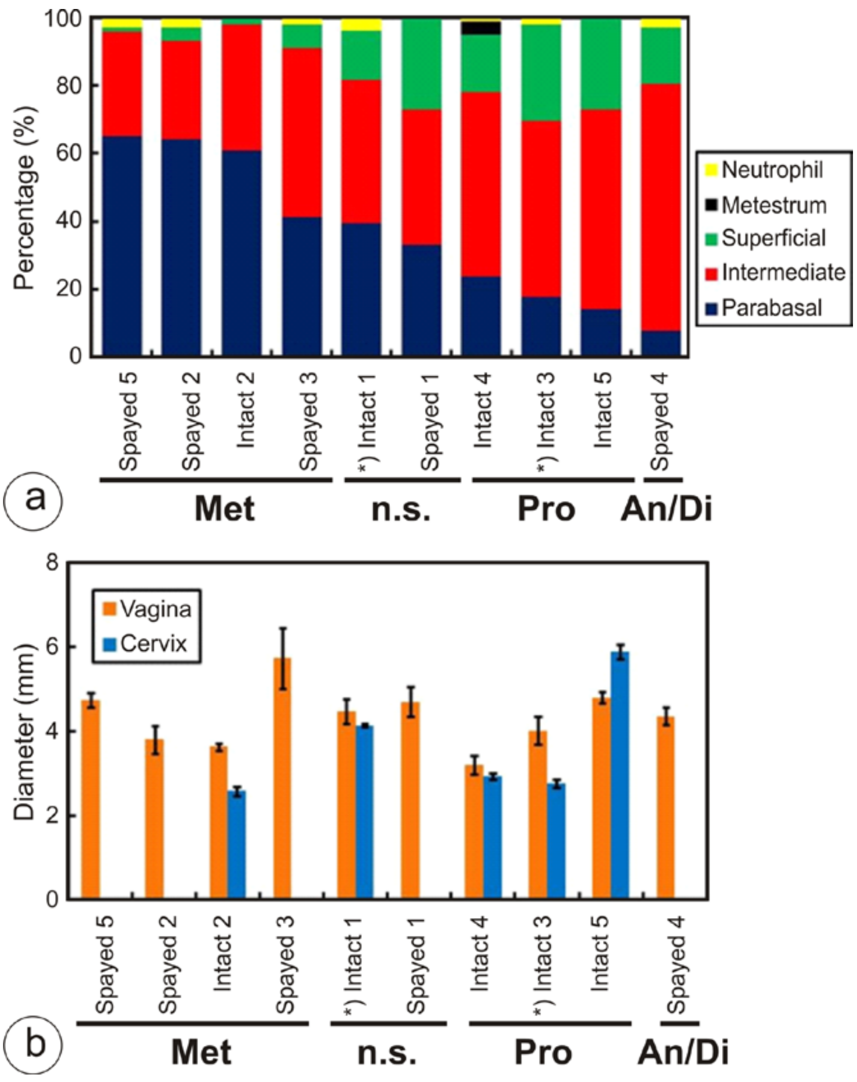

Figure 6. Status of estrus based on percentage of exfoliate cells that found in vaginal smears (a) and diameter of vagina and cervix (b). Note: Met=metestrus, n.s.=not specific, Pro=proestrus, An/Di=anestrus/diestrus, *)= multiparous.

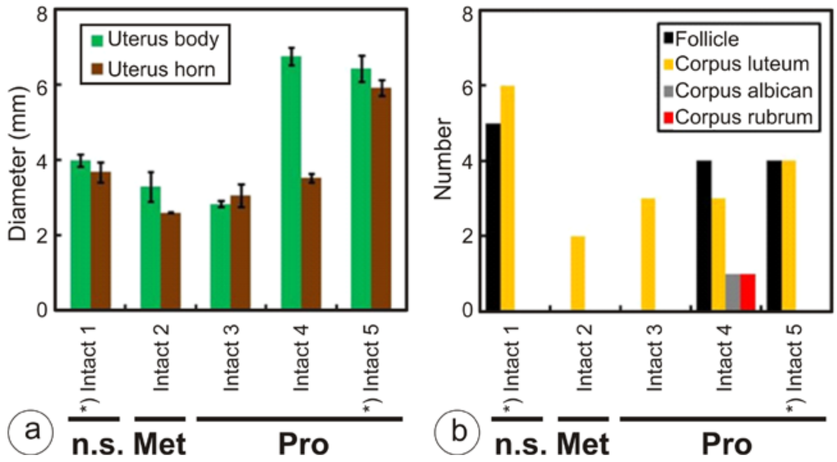

Figure 7. Status of estrus stage based on diameter of uterus (body and horn) (a) and number of follicle, corpus luteum, corpus albican, and corpus rubrum on ovary (b). Note: Met=metestrus, n.s.=not specific, Pro=proestrus, *)= multiparous.

Discussion

This study shows the complementing diagnosis between ultrasound imaging and vaginal cytology assessment in diagnosis of health reproductive status of Indonesian queens. The procedures were easily to be used in clinical veterinary practices as showed in Figure 1. Even tough, in this study only used 10 queens that consist of intact and spayed (Table 1), this study indicated that ultrasound and vaginal cytology can be used as mutual support in determining the phases of the estrus cycle. In general, the adult queen is a seasonally polyestrus and induced ovulator (Bristol-Gould & Woodruff 2006). Its ovulation is induced by coitus (Johnston et al. 1996). Coitus-induced ovulation is interpreted as the result of evolution to make sure that the ovulation will only happen after successful coitus, although it does not mean fertilization happens (Goodrowe et al. 1989). When the reproductive organs have reached maturity, the estrus cycle will happen in 5 stages, proestrus, estrus, interestrus, diestrus, and anestrus. The maturity of queen’s reproductive organs is influenced by its body weight and photoperiods (Feldman et al. 2014). Queens will sexually mature at the age of 4–12 months, usually between 6 or 8 months (Nutter et al. 2004). This is consistent with the results of this research which shows that 5-month-old queen Intact 5 had already shown ovarial activities by observing its follicles, corpus luteum, and corpus albicans (Table 4, Figure 4).

In sonogram, the shape of the vagina is the main characteristic to differentiate intact queens from the spayed ones. Vagina of spayed queens seems corrugated due to absent of the broad ligament (mesometrium, mesosalphynx, and mesovarium) that fixated and pulled the uterus cranially. The size of the front part of a spayed queen’s vagina is larger than intact one due to of the shortening of the spayed queen’s vagina, which makes the lumen vagina to be larger (Table 2, Figure 2b). As reported by (Davidson & Baker 2009), sonogram cervix seems like a thick, hyperechoic pipe in longitudinal view. In queens Intact 2, 3, and 4 which never give birth, the cervix’s diameter is smaller than queens Intact 1 and 5 (Table 2). It might be like in human (Sloane 2002), the difference in diameter of cervix in this study can be correlated with the nulliparous condition in queens Intact 2, 3, and 4.

Sonogram of the uterus body is appeared in expands from the end of the cervix to a third of the urine bladder’s length cranially (Figure 2c). The wall of uterus is consisted of three layers, which are the mucosa, the muscularis propria, and the serosa (Figure 2). Uterus can be distinguished from the intestines by the difference in appearing its wall layers. The instestinal wall is devided into four layers, which were mucosa, submucosa, muscularis propria, and serosa (Rao & Wang 2016), and then, the absence of peristaltic contractions, and its relatively straight course, unlike coiled nature the loops of the intestines (Mannion 2008). Endometrium and mesometrium are usually difficult to differentiate in sonogram (Figure 3, Table 3). The lumen of uterus can be seen hyperechoic because of mucus, or hypoechoic to anechoic if there is fluid fill in it (Davidson & Baker 2009). The outer part, hyperechoic layer is serosa (Gatel et al. 2016). The uterus is seen thicker in proestrus and estrus as the effects of the estrogen and the presence of fluid in it (Davidson & Baker 2009), as shown in Figure 3b.

The appearance ultrasonographic of ovary is varies based on the estrus stage of the queens (Figure 4, Table 4). Follicles are round shape, filled with fluid, and appear as anechoic in sonogram. The size of follicle in nulliparous queen is between 1–4 mm (Malandain et al. 2011), but in this study the follicle size are ranged between 2.21–5.82 mm. Graafian follicles are the large ovarian follicles with antrum folliculi which will ovulate later (Erickson 2000). Sonograms of the largest follicle size which can be reached before ovulation is 3.5 mm (Malandain et al. 2011), but in this study there are a lot of follicles having larger size. Corpus haemorrhagicum (corpus rubrum) is a follicle that was just ovulated, causing the empty antrum to be filled with blood thus causing it to be seen as a round structure with anechoic centre (Kurjak 1994). Corpus luteum is seen as a thick-walled structure, filled with fluid, and a hypo- or anechoic center (Davidson & Baker 2009; Mannion 2008). Corpus albicans (Figure 4a) is seen as a hyperechoic, hyalinated, and convoluted structure whose size is slowly getting smaller (Kurjak 1994).

Vaginal cytology (Figure 1, Figure 5) can be used to determine the stages of queen's estrus cycle (Mills et al. 1979). But according to (Malandain et al. 2011), this method is less precise if used to determine the stages of the ovary's follicle development. The ovary's follicle development is better viewed with ultrasound (Figure 4). However, all the queens that used in this study did not show any behavioral signs of estrus, supported by the ultrasound imaging of ovary (Table 4 and Figure 4) and vaginal cytology results (Table 5) showed that no queens were in estrus stage. Estrus is characterized by dominant amount of superficial vaginal epithelial cells (Mills et al. 1979). Vaginal smear of queens Intact 2 and Spayed 2, 3, 5 showed that those queens were in late metestrus (Table 5), which is characterized by the almost similar amount of parabasal and intermediate epithelial cells with a few superficial epithelial cells (Mills et al. 1979). These three queens (Intact 2, Spayed 2, 3, and 5) were suspected to have ovarian remnant syndrome (ORS). The ORS is a complication that happens because there are some ovarian tissues which were not taken off completely in an ovariohysterectomy procedure in spaying cats (Wallace 1991). The ovarian tissue remnants will undergo adhesion with the omentum, be vascularized and functional again which will then cause the estrus cycle to happen again (DeNardo et al. 2001).

Vaginal cytology of queens Intact 3, 4, and 5 results (Table 5) shows those queens were in proestrus stage which is characterized by the dominant amount of the intermediate epithelial cells and the almost-similar amounts of parabasal and superficial epithelial cells (Mills et al. 1979). The vaginal cytology result of queen Spayed 4 shows that the queen is in anestrus which is characterized by a very-dominant amount of intermediate epithelial cells (Feldman et al. 2014; Mills et al. 1979). The stage of the estrus of queens Intact 1 and Spayed 1 (Table 5) could not be determined based on the categorization of the vaginal cytology results by (Mills et al. 1979). This result's deviation may indicate of hormone-insufficiency or inflamed reproductive tract (vagina) problems (Mills et al. 1979). Neutrophils are normally found a lot in metestrus and a few in anestrus. Bacterial infection may cause neutrophils in large amount (fulfill of microscopic examination view) to be found in estrus and late proestrus (Mills et al. 1979). Neutrophils are usually not found in a queen which is in estrus, except there is an inflammation (vaginitis; fulfill of microscopic examination view). Normally, some bacteria can be found in queen's vaginal cytology assessment. This condition can be said as a pathological one if a high amount of neutrophils is also present (Valenciano & Cowell 2013). Metestrum cell is a vaginal epithelial cell that contains a neutrophil in its cytoplasm. This cell does not specifically characterize certain stage of estrus and can be found when regular neutrophils are also present (Cowell et al. 2007).

Even tough, reproductive hormone was not evaluated in this study, theoretically, the function of Gonadotropin Releasing Hormone (GnRH) which is generated in hypothalamus in low frequency to stimulate of Follicle Stimulating Hormone (FSH) release and in high frequency to stimulate releasing Luteinizing Hormone (LH) (Heffener & Danny 2008; Lüking-Jayes et al. 1997). The escalating amount of circulating estrogen hormone and development of follicle are happening in proestrus. The estrogen hormone also affects the start of superficial vaginal epithelial cell escalation in proestrus which can be seen in vaginal cytology of queens Intact 3, 4, and 5 (Table 5) until reaching its highest quantity to other vaginal epithelial cell types when estrus is happening (Rijnberk & Kooistra 2010). Concentration of estrogen hormone is decreasing when the follicles have reached maturity in a few days after estrus had started. Copulation will stimulate LH release which then will stimulate of ovulation. Mature follicles but non-ovulating will become atretic and decreases the concentration of circulating estrogen hormone. The concentration of circulating progesterone is still low as long as the estrus that happened did not result in ovulation (Engelking & Rebar 2012).

We also analyze the appearance of the sonogram of vagina and cervix size, and it was not affected by the statuses of estrus (Figure 6). Interestingly, the diameter of the uterus was affected by the ovary activity, where the ovary that owing follicle showed in large diameter compared to corpus luteum (Figure 7). As reported in llama by (Adams et al. 1989) that the uterus tone and shape was affected the ovary activity. Where, the tone and shape of uterus in follicular phase was higher than in luteal phase.

CONCLUSION

This study has successfully imaged the reproductive organs of queen by ultrasonography and their relationship to the results of vaginal cytology assessments. Combination of ultrasound and vaginal cytology diagnostic was able to strengthen the diagnosing the status of estrus in queens.

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Aflatoxin M1 in Fresh Dairy Milk from Small Individual Farms in Indonesia

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ABSTRAK

Widiastuti R, Anastasia Y. 2018. Residu aflatoksin M1 pada susu sapi segar dari peternakan sapi rakyat di Indonesia. JITV 23(3): 143-149. DOI: http://dx.doi.org/10.14334/jitv.v23i3.1789

Penelitan ini bertujuan untuk mengetahui keberadaan residu aflatoksin M1 (AFM1) pada susu sapi segar yang dikoleksi dari peternakan sapi perah perorangan. Sebanyak 104 sampel susu sapi segar dikoleksi dari Pangalengan-Bandung dan Sukabumi (propinsi Jawa Barat), serta Tanggamus (propinsi Lampung) pada bulan April dan September 2012. Semua sampel dianalisis secara kromatografi cair kinerja tinggi (KCKT) dan dideteksi dengan fluoresen detektor setelah dilakukan ekstraksi dengan pelarut organik. Kontaminasi AFM1 ditemukan pada 1,96% (1/51) dari sampel yang dikoleksi pada bulan April 2012 pada konsentrasi 1,20 ng/L dan 39,63% (21/53) dari sampel yang dikoleksi pada bulan September 2012 pada konsentrasi 1,0 – 34,1 ng/L. Sampel positif diperoleh dari sampel yang berasal dari Pangalengan dan Sukabumi, tetapi tidak ditemukan untuk sampel yang berasal dari Tanggamus pada pengumpulan bulan April maupun September 2012. Dari keseluruhan sampel positif AFM1 yang ditemukan, tidak ada sampel yang mengandung AFM1 melebihi batas maksimum (BM) yang diregulasikan di Indonesia (500 ng/L or 0.5 µg/L). Kontaminasi AFB1 pada kisaran 0,38 hingga 6,64 µg/kg ditemukan pada sampel pakan dari lokasi dan waktu pengumpulan yang sama. Temuan kontaminasi AFM1 pada susu segar yang dianalisis pada penelitian ini tidak membahayakan kesehatan manusia. Namun, pengawasan secara regular terhadap kontaminasi AFM1 pada susu dan AFB1 pada pakan sapi perah tetap diperlukan untuk menjamin kesehatan masyarakat.

Kata Kunci: Residu, Aflatoksin M1, Susu, Peternakan Rakyat, KCKT

ABSTRACT

Widiastuti R, Anastasia Y. 2018. Aflatoxin M1 residue in fresh dairy milk from small individual farms in Indonesia. JITV 23(3): 143-149. DOI: http://dx.doi.org/10.14334/jitv.v23i3.1789

This present study was aimed to investigate the presence of aflatoxin M1 (AFM1) residue in fresh dairy milk collected from small dairyl farms. A total of 104 samples of fresh cow's milk were collected in Pengalengan-Bandung and Sukabumi (West Java province), and Tanggamus (Lampung province) in April and September 2012. All samples were analyzed by a high performance liquid chromatography and detected with fluorescence detector after extraction with organic solvents. Contamination of AFM1 was found on 1.96% (1/51) from the samples collected in April 2012 at concentration of 1.20 ng/L and 39.63% (21/53) from the samples collected in September 2012 at concentration of 1.20 ng/L 1.0 – 34.1 ng/L. Those positive samples were obtained from Pangalengan and Sukabumi, but none for those samples collected from Tanggamus both on collection time April nor September 2012. In those positive samples for AFM1, there is no sample contained AFM1 above the maximum level (ML) regulated in Indonesia (500 ng/L or 0.5 µg/L). Low contamination levels of AFB1 in the range of 0.38 to 6.64 µg/kg found in supplemental feed samples from the same sampling time and locations. The findings of AFM1 contamination in raw fresh milk from this study caused no harm to the consumers. However, regular monitoring on the presence of AFM1 in dairy milk and aflatoxin B1 (AFB1) in dairy cattle feed is necessary to ensure the protection of human health.

Key Words: Residue, Aflatoxin M1, Milk, Small Individual Farms, HPLC

INTRODUCTION

Indonesia as a tropical country has a climate characterised by high humidity and high temperature which favours the growth of fungi in food products, and therefore the potential for the production of mycotoxins. Aflatoxins especially aflatoxin B1 (AFB1) isthe most toxic and carcinogenic mycotoxin. Aflatoxin B1 is metabolised into aflatoxin M_1 (AFM1) and excreted in milk (Agus et al. 2010)

There is a relation between food contaminated with aflatoxins and that fed to animals, and aflatoxin M1 in milk (Kang'Ethe & Lang 2009). About 0.3 to 6.2% of AFB1 in animal feeds is converted to AFM1, and it can be found in milk 12 hours after first ingestion and decreases to an undectable level 72 hours after las ingestionof AFB1 (Creppy 2002). Sumantri et al. (2012) revealed the conversion of AFB1 to AFM1 in Indonesia cattle was low (0.1%) compared than that in sub-tropical countries (around 1 to 3%). In addition, Agus et al. (2013) suggested that low conversion value

(0.32 to 0.82%) of Indonesian Friesian Holstein cattle is related to its low milk yield level. Mahmoudi & Norian (2015) suggested that the contamination levels of AFM1 in raw milk are dependent on the amount of AFB1 contained in feed animal (especially corn silage) to dairy cattle.

Aflatoxin M1 occurrence in dairy products may be considered as a possible hazard for public health and also can its residue be found in human breast milk (Kilic Altun et al. 2017). Its occurrence had been reported and reviewed from many countries. In 2012 a Working Group of the International Agency for Research on Cancer (IARC) finally concluded that there was sufficient evidence in humans for the carcinogenicity of aflatoxins (including aflatoxin M1) and classified as carcinogenic to humans (Group 1) (Ostry et al. 2017).

AFM1 was reported stable on pasteurization process or other milk processing such cheese making and storage (Fernandes et al. 2012; Sanli et al. 2012). Because of health concerns and regulatory limits, there is a Codex maximum level (ML) for a contaminant (including mycotoxins of AFM1) in a food or feed commodity, that is the maximum concentration of contaminant be legally permitted in that commodity as recommended by the Codex Alimentarius Commission (Codex Alimentarius 2015). The ML for AFM1 in milk is vary ranging from 0.02 to 0.05ng/mL depending on the country. The ML of AFM1 in Indonesia is 0.5 ng/mL (BSN 2009) which is similar to the MLs adopted in other Asian countries such as China, South Korea, Japan, Malaysia, the Phillipines, Singapore, Taiwan and Vietnam (Anukul et al. 2013). Additionally, the Indonesian regulation also governing the maximum limit of AFM1 in milk and milk based products (including pasteurized milk) at 0.5 ng/mL as stated by the Head of Indonesia National Agency of Drug and Food Control No. HK.00.06.1.52.4011 (BPOM 2009).

Fresh raw milk in Indonesia producedby cows that managed by individual smallholder farmers (owning about 3 to 10 cows). Milk arecollected to the local dairy center known as the dairy farmer cooperative (koperasi susu, Ind) before sent to the dairy processing companies. It is important to determine AFM1 levels in milk and dairy products in order to protect consumers of various age groups from its potential hazards. It is a necessitate for monitoring of AFM1 in fresh dairy milk before it processed further. Unfortunately, only few studies on AFM1 contamination in fresh dairy milk had been reported from Indonesia (Widiastuti et al. 2006; Nuryono et al. 2009). Therefore, the present study aimed to examine the the presence of AFM1 on raw dairy milk collected from small individual dairy farms in three different locations i.e Pangalengan-Bandung and Sukabumi (West Java), and Tanggamus (Lampung) in April 2012 and September 2012. This research was necessary to fulfill lack of supply data for the presence of AFM1 residue in fresh milk in Indonesia.

Mahmoudi & Norian (2015) suggested that the contamination levels of AFM1 in raw milk are dependent on the amount of AFB1 contained in feed animal (especially corn silage) to dairy cattle. Prandini et al. (2009) reported the most important risk factor for the AFM1 level in milk was the AFB1 concentration in supplemental feed component such as maize, groundnuts. Therefore the supplemental feeds such as cocoa pods, concentrate feed and rice hulls were also analysed for AFB1 to study its correlation with the occurrence of AFM1 in milk.

MATERIALS AND METHODS

Sample collection

A total of 104 fresh raw cow milk samples were collected in two different seasons, i.e at the end if wet season (April) 2012 and at the end of dry season (September) 2012. Those samples composed of 15 samples from Pangalengan (Bandung), 17 samples from Sukabumi and 19 samples from Tanggamus (Lampung) that collected in April 2012, and 14 samples from Pangalengan (Bandung), 19 samples from Sukabumi and 20 samples from Tanggamus (Lampung) that collected in September 2012. All samples were collected from small individual farms and carried out in the morning or evening milking time before were sent to dairy milk cooperative in every locations. Each sample of 200 mL in volume brought in a 250 mL plastic bottle, and transported to the laboratory in an ice box at temperatures about 4°C and then stored at -20°C until analysis of AFM1 residue.

A variety of supplemental feed samples for AFB1 analysis were also collected at the same time and same location of milk sampling. Those samples composed of 6 coconut cakes and 14 rice hulls, 3 cocoa pods and 2 cencentrate feeds were collected from Pangalengan (Bandung) and Sukabumi in April 2012. Whereas 17 rice hulls, 3 cocoa pods and 5 coconut cakes were collected from Bandung and Sukabumi in September 2012. None of supplemental feeds were obtained from Tanggamus (Lampung) in both sampling collection times. Each sample of 500 gr were brought in a plactic bag, and transported to the laboratory and then stored at -20°C until analysis.

Sample extraction for preparation of AFM1 analysis in milk

All milk samples were thawed gradually at 4°C and then vigorously mixed. The extraction method of AFM1 was modified from the Association of Analytical Chemists (AOAC) (Widiastuti et al. 2006). Ten mL of thawed milk sample was transferred into a 125 mL glass beaker containing 30 mL hot water (75°C) and shaked for 15 minutes. The sample was then filtered through a Whatman filter paper No. 41 and then slowly passed through to an SPE C_{18} (conditioned previously with 5 mL methanol and 5 mL deionized water) placed in a vacuum manifold. The cartridge was rinsed with a mixture of deionized water and acetonitrile (95:5, v/v) and the SPE C_{18} cartridge was removed and the inside of both stems were dried with tissue paper and was primed by adding 150µL acetonitrile to the inlet and let solvent soaked into packing for 30s. The SPE C18 cartridge was attached tandemly with an SPE silica column (activated previously with 5 ml ether) below the SPE C_{18} cartridge. Both cartridges were washed with 5 mL ether and the SPE C_{18} cartridge was released. The SPE silica column was rinsed with 2 ml ether. Finally, the AFM1 was eluted with 7 ml mixture of dichloromethane and ethanol (95:5, v/v) and the eluate evaporated to drynessand stored at -20°C in a freezer until further analysis.

Sample extraction for preparation of AFB1 analysis in feeds

All feed samples were also prepared for AFB1 analysis using the modified method (Widiastuti et al. 2008). Fifty gram sample was added with 200 mL mixture of methanol-water (85:15,v/v) and shaken for about 20 minutes. Forty mL of filtrate was extracted using 40 mL of 10% sodium chloride solution, 25 mL hexane and shake gently for 1 minute, and let the solution separated into 2 layers. The hexane layer (upper layer) was discarded and the filtrate (lower layer) was then extracted two timeswith 25 ml chloroform. The chloroform layer was passed through anhydrous sodium sulphate that placed upon a Whatman filter paper No. 41. The filtrate was evaporated and dissolved with 3 ml dichloromethane and then eventually applied to an SPE silica cartridge that previously had been activated with 3 ml hexane and 3 ml dichloromethane. After applying the sample, the cartridge was washed with 1 ml dichloromethane, 3 ml hexane, 3 ml anhydrate ether and 3 ml dichloromethane. The residue was eluted with 10 ml a mixture of chloroform and acetone (9:1, v/v). The eluate was evaporated to dryness and also stored at -20°C in a freezer until further analysis.

Chromatographic condition for detection of AFM1 and AFB1

All samples were derivatized before injected to the HPLC by adding 200 µL hexane and 50 µL trifluoroacetic acid (TFA) and evaporated to dryness on heating block at 100°C. A mobile phase was added into the derivatized sample and filtered through a 0.22 µm (pore size) and 13mm (diameter) PVDF filter (Waters Corp., USA).

Chromatographic condition for identification AFM1 and AFB1 was performedby using a column of C_{18} X-Terra RP_{18} (200 mm x 4.6 mm, 5 µm) (Waters Corp. USA) coupled with a guard column. The mobile phase for detecting AFM1 was a mixture of ultrapure water, acetonitrile and isopropanol (80:8:12, v/v/v), whereas for detecting AFB1 was a mixture of methanol, acetic acid and ultrapure water (15:20:65, v/v/v). All mobile phases were filtered through a 0.45 µm filter membrane, degassed and run isocratically at a flow rate of 1 mL/min. Twenty mL of the samples were injected onto the HPLC and detected with a Hitachi fluorescene detector model L 7485 (Hitachi Corp., Japan) which set at 425 nm emission and 365 nm excitation).

Chromatographic calculation

The extraction methods (AFM1 in milk and AFB1 in feed) were accredited according to ISO/IEC 17025. The quality of results is tested in each assay using a negative blank sample. The milk sample spiked with a known level of AFM1 (50 ng/L) in milk and AFB1 (40 ng/kg) in feed sample. Interpretation of positive samples were taken based on the value given by the chromatograms that higher than the quantitation limit obtained on the methods. The quantification limit is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy by using the developed HPLC method (Shrivastava & Gupta, 2011). The quantitation limits were 1.0 ng/L for AFM1 in milk and 1 µg/kg for AFB1 in feed. A calculation result **above the quantitation limit** gives a meaning as **positive sample**, whereas **lower than the quantitation limit** gives a meaning as trace or **not detected (ND)**.

RESULTS AND DISCUSSION

Contamination of AFM1 in raw milk samples

The appearance of AFM1 in milk is within 15 minutes to an hour after the cows consuming AFB1 and decrease to below the limit of detection within 72 hrs and returns to baseline levels within two to three days after AFB1 removed from the diet (van Egmond 1989). Agus et al. (2010) suggested that AFM1 rapidly appeared in milk in the range of 0.08 to 0.20% of the AFB1 consumed by the from Indonesian Frisian Holsteins (FH) and still detected until 5 days after AFB1 was removed from the diet.

Analysis results for the occurrence of AFM1 contamination in dairy milk from 3 different locations (Pangalengan-Bandung, Sukabumi, and Tanggamus) collected in April 2012 and September 2012 presented in Table 1 and 2. Contamination of AFM1 found in 1.96% (1/51) of the samples collected in April 2012 at a concentration of 1.20 ng/L and 39.63% (21/53) of the samples collected in September 2012 at a concentration level of 1.0 - 34.1 ng/L for samples collected from Pangalengan and Sukabumi, but not found in samples from Tanggamus neither collected in April nor September 2012. The highest concentration of AFM1 at 34.1 ng/L was found in one sample from Sukabumi collected in September 2012. Totally, the incidence of AFM1 contamination is 21.15% (22 out of 104 samples) with the concentration range of 1 to 34.1 ng/L. None of those samples had AFM1 greater than the maximum level (ML) regulated in Indonesia (500 ng/L or 0.5 µg/L).

Those data resulted in this study shows a better comparison to the previous research conducted from the same location in Pangalengan-Bandung in 2003, which was reported that contamination of AFM1 occurred in 85% among 20 samples in the level range of 2 to 1200 ng/L (Widiastuti et al. 2006). Fillaeli (2007) observed 95% of milk samples from Yogyakarta were contaminated with AFM1 at concentration level of 33 to 113 ng/L, Nuryono et al. (2009) reported 70% of the samples observed contaminated with AFM1 at concentration level ≤10 ng/L and Nurhayati (2014) found 54 out of 57 pasteurised milk were consisted of AFM1 at concentration level of 20.77 to 458.87 ng/L. Aflatoxin M1 also was analysed on 20 powdered milk collected from Serang, Bandung, Semarang and Surabaya, and had been reported in the range from undetectable to 0.549 µg/kg and the highest data (55%) was distributed in concentration range of >0.05 µg/kg to 0.2 µg/kg (Wijaya et al. 2018).

Our results were better compared to the study conducted by Nadira et al. (2017) in Malaysia who revealed 19 out of 53 samples (35.8%) were positive with AFM1 ranging from 3.5 to 100.5 ng/L for detection with ELISA method or the study conducted by Ruangwises & Ruangwises (2010) in 240 milk samples collected from central region of Thailand which showed the average concentration in winter was 89±34 ng/L, rainy season was 71±28 ng/L and summer was 50±21 ng/L, and also the study conducted by Goncalves et al. (2017) who found that 40.4% of samples from small dairy farms in Brazil were above the maximum limit allowed by the Brazilian regulation (0.5 µg/L). In the other hand, our results showed a higher incidence compared to the results in Malaysia (4% of the analyzed 102 samples) at contamination levels below of 0.5 ng/kg) (Shuib et al. 2017).

AFB1 contamination in supplemental feeds

The levels of AFM1 in milk are influenced by both feeding practices and the types of feedstuffs. The feed supplied to dairy herds in those collection sample regions was predominantly fresh grasses and sometimes mixtured with concentrate or rice hull. Table 3 and 4 present the occurrence of AFB1 in supplemental feeds (coconut cake, rice hull, cocoa pods, concentrate feed) from every locations of milk sampling conducted.

Table 1. The occurrence of AFM1 in raw fresh milk collected in April 2012

Sampling locations	Sample size (N)	AFM1 contamination	
		Incidence, n (%)	Concentration (ng/L)
Pangalengan	15	1 (6.66%)	1.20
Sukabumi	17	none	-
Tanggamus	19	none	-
Total	51	1 (1.96%)	1.20

Table 2. The occurrence of AFM1 in raw fresh milk collected in September 2012

Sampling locations	Sample size (N)	AFM1 contamination	
		Incidence, n (%)	Concentration (ng/L)
Pangalengan	14	7 (50.0%)	5.3 - 34.1
Sukabumi	19	14 (77.8%)	1.0 - 33.2
Tanggamus	20	none	-
Total	53	21 (39.63%)	1.0 - 34.1

The contamination levels of AFB1 in all types of supplemental feed samples were very low (0.38 to 6.64 µg/kg). The highest incidence (100%) of the presence of AFB1 was found in coconut cake samples collected from Pangalengan in sampling time April 2012 in the average concentration of 4.05 µg/kg

The results on the presence of AFB1 in supplemental feed as presented in Table 3 and 4 are in give strong correlation with low incidence and low contamination levels of AFM1 in milk presented in Table 1 and 2 (ranging 1.0 to 34.1 ng/L). The most probably reason of low incidence on the presence of AFM1 in April 2012 sampling time might be due to the types of feed given to those animals were fresh grass which were not suitable for the growth of *A. flavus* and/or *A. parasiticus*. The results of this study in agreement to the finding of Picinin et al. (2013) in Brazil who reported that rainy season caused less of no AFM1 contamination due to widely available of grass and cattle consumed less concentrate feed.

In contrast, AFM1 presents in 39.63% of samples collected in September 2012 (the end of dry season) indicates that during that time the availability of fresh grass is very limited so that farmers use supplemental feed to fulfill the nutritional needs of cows which in turn lead to the presence of AFM1 in the milk produced, and in agreement with the investigation done by Bilandzic et al. (2017) in Croatia who found that extremely hot temperature during summer and long period of drought without rain may stimulate the development of toxigenic mould to synthezise AFB1 to AFM1 and increased the incidence and the concentration of AFM1. These findings, differ to the investigation conducted in Iran by (Mahmoudi & Norian 2015) who found that the average contamination levels of AFB1 from summer were lower than from winter.

Low concentration of AFB1 in supplemental feed supports the fact of low concentration of AFM1 in milk samples. Mahmoudi & Norian (2015) found 82.40% (178/216) of corn silage, alfalfa hay and concentrate samples from difference dairy farmers were positive for AFB1 in the average AF level of 1.55±0.89 µg/kg and mostly related to the corn silage, and 36.51% (65/178) of the samples had AF levels that exceeded 5 µg/kg.

Since that the contamination levels of AFM1 in raw milk are dependent on the amount of AFB1 contained in feed, it is important also to consider the maximum limit of AFB1 in feed destined for dairy cattle. The maximum limit of AFB1 in Indonesia according to SNI 3148.1:2009 is 200 µg/kg (BSN 2009) is too high compared to the recommendation by Food and Drug Adminstration (FDA) in United States of 20 µg/kg (FAO 2004) by assuming the average of transfer rate of AFB1 to AFM1 is 66:1 (or equal to 300 ng/L of AFM1).

Table 3. The occurrence of AFB1 in supplemental feeds collected in April 2012

Sampling locations	Sample types	Sample size (N)	AFB1 contamination	
			Incidence, n (%)	Average concentration (µg/kg)
Pangalengan	Coconut cake	6	6 (100%)	4.05
	Rice hull	8	3 (37.5%)	4.18
Sukabumi	Rice hull	8	3 (37.5%)	0.45
	Cocoa pods	3	2 (66.6%)	0.38
	Concentrate feed	2	1 (50.0%)	0.99
Tanggamus	NA	-	-	-

NA = not available

Table 4. The occurrence of AFB1 in supplemental feeds collected in September 2012

Sampling locations	Sample types	Sample size (N)	AFB1 contamination	
			Incidence, n (%)	Average concentration (µg/kg)
Bandung	Rice hull	15	6 (44.4%)	6.64
Sukabumi	Rice hulls	2	-	ND
	Cocoa pods	3	1 (33.3%)	0.53
	Coconut cakes	5	1 (20.0%)	3.41
Tanggamus	NA	-	-	-

NA = not available; ND = not detected

CONCLUSION

Contamination of AFM1 was found on 1.96% (1/51) from the samples collected in April 2012 at concentration of 1.20 ng/L and 39.63% (21/53) from the samples collected in September 2012 at concentration of 1.20 ng/L 1.0 – 34.1 ng/L. Those positive samples were obtained from Pangalengan and Sukabumi, but none for those samples collected from Tanggamus both on collection time April nor September 2012. In those positive samples for AFM1, there is no sample contained AFM1 above the maximum level (ML) regulated in Indonesia (500 ng/L or 0.5 µg/L). Low contamination levels of AFB1 in the range of 0.38 to 6.64 µg/kg found in supplemental feed samples from the same sampling time and locations.

The findings of AFM1 contamination in raw fresh milk from this study caused no harm to the consumers. However, regular monitoring on the presence of AFM1 in dairy milk and aflatoxin B1 (AFB1) in dairy cattle feed is necessary to ensure the protection of human health.

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Nutrition Quality and Microbial Content of Buffalo, Cow, and Goat Milk from West Sumatera

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ABSTRAK

Melia S, Yuherman, Ferawati, Jaswandi, Purwanto H, Purwati E. 2018. Kualitas nutrisi dan kandungan mikrobiologi pada susu kerbau, sapi dan kambing dari Sumatera Barat. JITV 23(3): 150-157. DOI: http://dx.doi.org/10.14334/jitv.v23i3.1594

Penelitian ini bertujuan untuk mengetahui kualitas susu segar secara fisik, kimia dan mikrobiologis yang diperoleh dari ternak sapi, kambing dan kerbau di Sumatera Barat. Parameter yang diukur untuk menentukan kualitas susu segar adalah kandungan nilai gizi, total koloni bakteri aerob dan bakteri asam laktat serta jenis bakteri asam laktat. Hasil penelitian menunjukkan kandungan nilai gizi susu telah memenuhi Standardisasi Nasional Indonesia, tetapi total koloni bakteri aerob berada diatas ambang batas yang diizinkan yaitu > $1x10^6$ CFU/ml. Di samping itu, setiap sampel memiliki total koloni bakteri asam laktat yang bervariasi. Nilai total bakteri asam laktat (BAL) terendah diperoleh pada susu sapi yaitu 0,84 ± 0,18 $x10^7$ CFU/ml, berbeda halnya dengan susu kerbau dan susu kambing yang memiliki total BAL yang lebih tinggi yaitu 36,8 ± 17,57 $x10^7$ CFU/ml dan 57,25 ± 8,89 $x10^7$ CFU/ml. Namun semua koloni memperlihatkan morfologi isolat BAL yang hampir sama. Dari hasil penelitian disimpulkan bahwa susu segar dari Sumatera Barat mengandung BAL meskipun masih diperlukan pengawasan sanitasi selama penanganan susu.

Kata Kunci: Susu Segar, Nutrisi, Total Plate Count, Bakteri Asam Laktat

ABSTRACT

Melia S, Yuherman, Ferawati, Jaswandi, Purwanto H, Purwati E. 2018 Nutrition quality and microbial content of buffalo, cow, and goat milk from West Sumatera. JITV 23(3): 150-157. DOI: http://dx.doi.org/10.14334/jitv.v23i3.1594

The aim of this research was to determine the quality of fresh milk physically, chemically and microbiologically obtained from cow, goats and buffalo in West Sumatra. The research method applied was laboratory experimental to analyze nutritional value, the number of aerobic bacteria and lactic acid bacteria, isolating and identifying lactic acid bacteria. Results showed that the nutritional value of milk had meet the requirements of Indonesian National Standardization, but the total colony of aerobic bacteria was above the allowed threshold of 1 x 10^6 CFU/ml. In addition, each sample had a total colony of varied lactic acid bacteria (LAB). The lowest total LAB value obtained in cow's milk was 0.84±0.18 $x10^7$ CFU/ml, in contrast to buffalo milk and goat milk which had a higher total LAB of 36.8±17.57 $x10^7$ CFU/ ml and 57.25±8.89 $x10^7$ CFU/ml. However, all the colonies showed almost identical morphology of LAB isolates. It is concluded that fresh milk from West Sumatra contains LAB therefore sanitation control is still needed during handling of milk.

Key Words: Fresh Milk, Nutrients, Total Plate Count, Lactic Acid Bacteria

INTRODUCTION

West Sumatra is a potential area for the development of livestock business. Various livestock business have developed rapidly from year to year. It shows significant increase in production of livestock main products such as egg, meat and milk. Milk production not only comes from cow but also from goat and buffalo. Padang Panjang city is the largest milk supplier in West Sumatera with the highest production of around 405 ton/year (Animal Husbandry and Animal Health Service of West Sumatera Province 2014).

Fresh milk is a liquid derived from healthy and clean cows, obtained by proper milking, which its natural content is not reduced or added by anything and has not received any treatment except cooling (BSN 2011). Milk contains chemical elements needed by the body such as protein and high fat. The main constituents of milk are water (87.9%), protein (3.5%), fat (3.5-4.2%), vitamins and minerals (0.85%). The pH value of milk between 6.5 to 6.6 is a very favorable condition for microorganisms, where the pH around 6.5-7.5 is best for bacterial growth causing milk to easily damage (Estiasih & Ahmadi 2009). The complete nutritional content of milk becomes a food sources for microbes that cause easly damage of milk. The main damage to milk is caused by the activity of microorganisms that can be a source of disease for human who consume it. Proper milk handling is crucial to reduce the contamination and safe for consumption.

High milk production should be supported by good milk quality. Good quality milk is characterized by physical, chemical and microbiological quality. This is a crucial concern because milk is high nutritional food preferred by microorganisms to grow that leads to easily deteriorated milk. The contaminated milk will harmful to the consumer such as causing diarrhea. This disease is caused by the contamination of disease-causing microbes such as Coliform (*Escherechia coli*) and Staphylococcus aureus in an amount exceeding the limit of >2 x 10^1 (Indonesian National Standard 2000). Usually, these two types of bacteria come from the surrounding environment. This kind of disease is usually called food borne disease. Donkor et al. (2007) described that Coliform bacteria can develop rapidly in humid milk residues in the container, which then becomes the main source of milk contamination. Furthermore, Tamime (2007) explained that pathogenic organisms in milk is bovine mastitis and externally contaminating milk. Bacteria that cause mastitis, which varies geographically and with different livestock practices including *S. aureus*, *Streptococcus agalactiae*, *Str. dysgalactiae*, *Str. uberis*, *Listeria* spp. and *E. coli*., *S. aureus*, producing heat-resistant enterotoxins that can cause food poisoning. The *Str. agalactiae* causes bacteremia and meningitis, which are potentially fatal for infected infants. The *Salmonella* bacteria and Campylobacter thermoduric strains are the most common pathogens that originate from the external of the udder.

High quality of milk can be seen from the value of its nutritional components such as of water, protein, fat, lactose and total solid. Besides, it also can be from the amount of aerobic and anaerobic bacteria contained in milk, seen as microbiological aspect. The purpose of this activity was to study the quality of nutrients and microbiology of buffalo, cow and goat milk from West Sumatra.

MATERIALS AND METHODS

The materials used in this study were: fresh milk (from cows, goats and buffaloes) obtained from five locations, Payakumbuh, Sijunjung, Tanah Datar, Padang Panjang and Solok in West Sumatra; Man Rogossa sharpe Agar (MRSA) (Merck); MRSB (Merck); Nutrient Agar (NA) (Merck); PCA (Plate Count Agar), pepton water; destlilled water; alcohol; and spritus. The equipments used were laminar flow (AlabTech), cool box (Coleman), thermometer, pH meter (HANNA instrument pH meter), petri dish, ose needle, incubator (INFORS AG-CH-4103 BOTTMIGEN), measuring cup, analytical scales KERN ABT 320-4M), erlenmeyer, bunsen lamp, test tube, beaker glass, dropper drip, hockey stick, autoclave, centrifuge (5417 R), vortex, spectrophotometer (Shimadzu UV-1800 series), oven (Memmert) and micro pipette (Thermo). The research was conducted in Animal Production Technology Laboratory and Biotechnology Laboratory-Faculty of Animal Husbandry, the University of Andalas during January - June 2017.

Those cow, goat and buffalo milk collected from farms were stored in cool boxes until samples were analyzed in the laboratory. A total 15 samples collected randomly at three sites in each location, with 3 repetitions. Milk was milked from 4-6 years old goat, of 3.5-5 years old cow and 3-4 years old buffalo. The milk collection was done during milking in the morning at 06.00 - 08.00 WIB.

Proximate Analysis. Proximate analyzes performed were test of water content, protein, fat, lactose, total solid, and pH (AOAC 2012)

Total colonies of aerobic and lactic acid bacteria. To calculate total colony of aerobic lactic acid bacteria was done according to Purwati et al. (2005). Calculation of the number of colonies of aerobic bacteria contained in the sample was done by the following procedure: All equipment needed was sterilized first with autoclave at 121°C for 15 minutes with 15 lb pressure. The medium used was 23.5 gram Plate Count Agar (PCA) that dissolved with 1000 ml of aquades, 20 grams of pepton water dissolved with 1000 ml of aquades, then homogenized with magnetic stirrer above hot plate at 100°C and sterilized in autoclave. A total of 1 ml sample that was dissolved in 9 ml pepton, then was vortexed for 5 minutes until the average yield called 10-1 dilution. The dilution was then taken 100 µl which already contained 900 µl pepton water solutions. The result was called 10-2 dilution. And so on until 10-7 dilution. Furthermore, the total of 100 µl of the 10-7 was taken to be spread on petridish that has contained frozen PCA. The petridish was stored in an incubator for 24 hours at 37°C which has already been coded. After 24 hours, bacterial colonies grown were calculated using the Quebec Colony Counter (Colony Forming Unit) tool. The number of colonies was multiplied by 10 that formed in the calculation formula bellow::

$$\text{CFU/gram} = \textit{The number of colony} \times \frac{1}{\textit{Dilution}} \times \frac{1}{\textit{The weight of sample}}$$

The calculation of total LAB of Purwati et al. (2005) was prepared by sterilizing all the equipment in the autoclave at 121°C for 15 minutes at a pressure of 15 lbs. Media prepared was De Mann Ragosa Sharpe (MRS) Broth (Merck). A total of 1 ml of milk was dissolved in a reaction tube containing of 9 ml of de mann Ragosa Sharpe (MRS) Broth solution, then vortexed until homogeneous. The result was called 10-1 dilution, then it was incubated for 24 hours in an incubator at 37°C. A total of 100 µl of the dilution was inserted into an eppendrof tube containing of 900 µl de mann Rogosa Sharpe (MRS) Broth solution, then vortexed until homogeneous. The result of this dilution was called dilution 10-2, and so on until it formed dilution 10-8. It is taken 100 µl of dilution 10-8 and spread on petridish which already contain MRS media so that then flattened with hokey stick which have been sterilized by alcohol and burned with Bunsen then aerated. The inoculum was stored in an anaerobic jar and incubated in an incubator for 48 hours at 37°C. The petridish was marked as code. After 48 hours, the single colony that had characterizes of the LAB such as yellowish smooth round was calculated and inserted into the same formula as the TPC formula above.

Isolation and identification of lactic acid bacteria

Morphology of lactic acid bacteria is known macroscopically and microscopically and followed by biochemical test (gram staining and catalase test) in accordance to Purwati et al. (2005).

a. Macroscopic: Visual observation done on shape, size, color, and elevation
b. Microscopic: Shape of cell using microscope 400x and 1000x
c. Biochemistry (Color Staining and Cathalase Test)

Data analysis

Data processing was done by determining the average obtained from 3 repetitions on proximate and microbiological analysis then it was counted the standard deviation.

RESULTS AND DISCUSSION

Proximate analysis of milk

The proximate test is part of the method to determine the quality of milk. The tests included determination of water content, protein content, fat content, lactose and total solid. Results of proximate analysis of buffalo, cow and goat milk is presented in Table 1.

The analysis result showed that the average water content in buffalo milk was 78.91%. This is lower than the research of Han et al. (2012) that showed buffalo milk content was around 83%. The average water content of cow milk in West Sumatra (80.82%) was lower compared to Taufik (2004) that was 87.89%. This can be caused by the high fat content of cow's milk leads high total solid milk. Another factor that also affects the milk content is the type and amount of feed given. This is in accordance with Lingathurai et al. (2009) who stated that the physical and chemical quality of fresh cow's milk is influenced by dairy breed, feed, feeding, milking frequency, milking method, seasonal and the location when lactation.

Protein content of buffalo, goat and cow milk obtained from various breeders in West Sumatra is presented in Table 1. Buffalo milk protein is 6.77% that higher than the buffalo milk protein content in the study of Wirdahayati (2006) of 5.62%. The protein content of cow milk in this study (3.71%) is also higher compared to cow milk protein content in Suhendar et al. (2008) of 3.2%. While the Indonesian National Standard (INS) Number 01-3141-2011, the minimum protein content in fresh cow's milk is 2.8%. It means that the cow milk has met the INS. Furthermore, protein content of goat milk in this study is 4.39% higher compared to research Watson et al. (2017) of 3-4%.

Table 1. Average of proximate analysis of buffalo, cow, and goat milk in West Sumatera

Milk	The number of sample	Water level (%)	Protein level (%)	Fat level (%)	Lactose (%)	Total solid (%)	pH
Buffalo milk	45	78.91±0.75	6.77±0.31	7.25±0.16	5.28±0.12	19.31±0.28	6.60±0.14
Cow milk	45	80.82±0.05	3.71±0.32	5.21±0.23	4.34±0.26	13.26±0.35	6.40±0.06
Goat milk	45	82.21±0.89	4.39±0.22	6.41±0.36	4.58±0.07	15.64±0.48	6.40±0.16
INS			Min 2.8%	≥ 3%	3.00-5.80 mg/100 ml	12-13%	6-7

High level of protein in goat's milk is expected to be influenced by the animal feed itself. Generally, the management pattern of goats in West Sumatra has begun to pay attention to the adequacy of nutritional value. It is not only sourced from grass as forage but also the adequacy of concentrate for goats. This is in line with the Melia & Sugitha (2007) who stated that the concentrate is a feed ingredient that has a complete nutritional content, so it will affect the amount of solid of non-fat content in milk including protein.

The fat content of milk in this study was in the higher range than the minimum limit in the Indonesian National Standard/Badan Standarisasi Nasional (INS/BSN) Number 01 01-3141-2011 about - Terms of Quality Fresh milk that is at least 3%. In this research, the fat content of buffalo, cow, and goat milk was 7.25%, 5.21%, and 6.41%, respectively. Factors affecting milk fat levels are animal breed maintained, milking age, lactation levels, milking intervals, local climatic conditions and diet given. If the dominant diet given is forage, the fat content in milk will be high due to the crude fiber consumed will be fermented by rumen microbes to produce acetic acid as the basic ingredient of milk fat formation (Tyler & Ensminger 2006). The lactose content of buffalo, cow and goat milk in this study was 5.28%, 4.34%, and 4.58% respectively. Normal milk lactose content is about 4% (Sinuhaji 2006). The lactose content of cow, goat and buffalo milk in this study was already in the range of normal values.

The composers of total solids in milk are proteins, fats, lactose, vitamins, and minerals. According to Haenlein (2002), the total solid milk content is 12-13%. The total solid content of goat's milk in this study was 15.64% higher than the normal total solid. The total solid value of milk is influenced by the fat content of milk, solid nonfat and specific gravity of milk. The total solid content is highly dependent on fat content and solid nonfat. If you see total solid content of buffalo milk was higher than cow's milk (19.31%) while cow milk was 13.26%. Buffalo milk has average solid total of 16-18% (Han et al. 2012). The total solid of buffalo milk of this study is similar to Kapadiya et al. (2016) showed that total solid milk buffalo is higher than the total solid of cow and goat milk.

The pH of milk on the Indonesian National Standard (INS) Number 01-3141-1998 about Quality Requirement Fresh Milk is ranged around 6-7. When the pH of milk is higher than the normal limit it can usually be interpreted that the animal is exposed to mastitis. While, when it is below than 6.5, it can be caused by bacteria or is a sign of milk colostrum (Watson et al. 2017). The pH of milk in this study (Table 1) shows that milk in West Sumatra is in normal category. Most of the acids present in milk are lactic acid. Based on the nutrient content and pH value, those milk sample collected in West Sumatra already meets the standards according to INS (Indonesian National Standard).

Goat milk contains higher unsaturated fatty acids and more medium chain triglycerides than cow and buffalo milk that good for health, especially for the heart by lowering blood cholesterol levels (Haenlein 2004). The content of cholestrerol in goat milk of 12 mg/100 ml was lower than cow milk of 17 mg/100 ml. Besides, fatty acids in goat milk such as caprylate is able to remove dead skin cells, it is why goat milk is widely used for the manufacture of high economic value-cosmetic materials (Alo 2008).

Total colony of aerob and lactic acid bacteria of milk

The microbiological characteristic as a parameter of milk quality is based on the amount of aerobic bacteria contamination. However, the amount of lactic acid bacteria indicates the potential of milk as probiotics. The analysis result of the total colonies of aerobic and lactic acid bacteria in fresh milk in West Sumatra is presented in Table 2.

The results of TPC calculation of buffalo, cow, and goat milk can be seen in Table 2. It shows that milk in West Sumatera exceeds the limit of TPC threshold set by the INS. Indonesian National Standard (INS) Number 01-3141-1998 is on the Quality Requirement of Fresh Milk and INS Number 7388: 2009 is on the Maximum Limit of Microbe Contamination in Food.

Table 2. Average total colony of aerob and lactic acid bacteria of buffalo, cow and goat milk in West Sumatera

Sample	The number of sample	Total LAB ($\times 10^7$)	TPC ($\times 10^5$)
Buffalo milk	45	36.8±17.57	296±16.63
Cow milk	45	0.84±0.18	33±11.61
Goat milk	45	57.25±8.89	116.5±7.73
INS	-		$\leq 1 \times 10^6$ CFU/ml

The INS suggests that bacterial or microbial contamination is 1 x 10^6 CFU/ml of Total Plate Count/TPC. The results of this study did not differ much from the results of Zeinab et al. (2008) that showed an average TPC values in cow's milk in Sudan state of 7.98 log/ml. This illustrates the lack of sanitation in the management of animal management and post-milk handling.

High TPC value obtained in West Sumatera is allegedly caused by the manual milking and handling process carried by the farmers. A semi-permanent cattle enclosure, the sewer that close to the milking site and no special milking place cause easy milk to be contaminated by the fecal bacteria. Dairy milking equipment is one of the causes of high TPC in milk in West Sumatra. During the research it was informed that milking was usually done in open cages that allow for contamination through the air, equipment or cattle body (Donkor et al. 2007). Poor hygiene in this study indicated the presence of milk contamination from the milking environment.

High TPC values in cow, goat and buffalo milk show a contamination in milk both from outside and other sources. Contamination in milk by pathogenic or non-pathogenic bacteria may come from the cow itself, milking equipment, not clean storage room, dust, air, flies, and wrong handling by humans (Roumbaut 2005). According to Cempirkova (2006), a total of 64% of microorganisms in milk come from poor hygiene, 28% by low temperatures (psychotrophic bacteria) and poor storage, and 8% by mastitis. It is supported by Lues et al. (2010) who stated that cows with mastitis may lead the number of TPC to reach 1 x 10^7 CFU/mL. There are two types of mastitis in dairy cattle: clinical and sub-clinical mastitis. Clinical mastitis is characterized by the changes in milk that looks clot or liquid and mixed with blood or pus. While sub-clinical mastitis cannot be observed directly so it is necessary to test using a special mastitis reagent (Sudono et al. 2003). However, direct observation of animal conditions in this study did not indicate the symptoms of clinical mastitis. According to Ortolani et al. (2010), the presence of aerobic microbes is often regarded as an important microbiological factor for the quality parameters of milk and dairy products at an amount of higher than 10^5 CFU/mL so as to indicate the cleanliness of production, while the values that lower than 20,000 CFU/mL shows good sanitation practices.

Table 2 shows that in fresh cow, goats and buffalo milk there are also lactic acid bacteria. Rizqiati et al. (2015) said that milk from various types of mammals can be used as a source of Lactic Acid Bacteria (LAB) and buffalo milk is the source of various potentially probiotic LABs. Kumar & Murugalatha (2012) found a species of lactic acid bacteria, Lactobacillus plantarum isolated from cow's milk that has the ability to kill various types of pathogenic bacteria. Lactic acid bacteria are widely spread in nature. It naturally occur due to the original microflora in raw milk is a very active gram-positive bacteria in food processing and feed fermentation (Guessas & Kihal 2004). Then, Ortolani et al. (2010) stated that the LAB can be used as biopreservative in food. Lactic acid bacteria are gram-positive, non-spore, spherical or stem bacteria that produce lactic acid as the final product of carbohydrate fermentation.

Lactic acid bacteria (LAB) can be isolated from various natural sources, such as from soil; plants; water; sewage and also from the genital tract and human and animal digestion (Sujaya et al. 2016). The current utilization of LAB is not only to produce food as it has been widely used as fermented starter for the manufacture of cheese, yoghurt and curd but the LAB is used as a probiotic that has many functions, especially plays an important role in the digestive tract. In line with Noordiana et al. (2013) who said that lactic acid bacteria are also classified as probiotic bacteria which have antimicrobial activity against certain microorganisms. While Kim et al. (2006) stated that BAL is also tolerant of stomach acid and harmless.

Isolation and identification of lactic acid bacteria

Identification of lactic acid bacteria isolates using morphological features, as well as gaseous formation of glucose can only group the LAB as *Lactobacillus* spp. (for isolates with rod shape), *Pediococcus* spp. (for isolates with round/coccus), *Lactococcus/Enterococcus* spp. (for isolates with cocco bacil form) (Sujaya et al. 2016). Morphology characteristic of isolates of LAB is known by gram staining.

The identification of LAB in fresh milk obtained from West Sumatera region was done through several conventional test stages, namely the determination of isolate morphology, gram staining test and catalase test grouped as Gram-positive bacteria. Melia et al. (2017) stateed that LAB isolated from buffalo milk has potential as probiotics and inhibits the growth of pathogenic bacteria, including Lactobacillus fermentum. The result of identification of lactic acid bacteria conventionally in this study is presented in Table 3.

The principle of LAB isolation is to obtain a single colony to determine the properties of LAB. LAB identification is indicated by gram staining and catalase test and morphological characteristics of each isolate (shape, size, elevation, color). Gram staining test result showed purple-colored and tube-shaped. This shows that the isolates tested are gram positive. While the catalase test on all isolates did not show any gas bubbles after spilled with hydrogen peroxide (H_2O_2).

Table 3. Morphology of lactic acid bacteria of fresh milk in West Sumatera

Sample	Isolate	Shape	Size	Color	Elevation	Gram staining	Catalase test
Buffalo milk	BM 1.1.	Tube	3 mm	Beige	Convex	Positive	Negative
	BM 2.1	Tube	3 mm	Beige	Convex	Positive	Negative
	BM 3.2	Tube	3 mm	Beige	Convex	Positive	Negative
	BM 4.2	Tube	2 mm	Beige	Convex	Positive	Negative
Cow milk	CM 1.1	Tube	2 mm	Beige	Convex	Positive	Negative
	CM 1.2	Tube	3 mm	Beige	Convex	Positive	Negative
	CM 2.1.	Tube	2 mm	Beige	Convex	Positive	Negative
	CM 2.2	Tube	3 mm	Beige	Convex	Positive	Negative
Goat milk	GM 1.1	Tube	2 mm	Beige	Convex	Positive	Negative
	GM 2.1	Tube	1 mm	Beige	Convex	Positive	Negative
	GM 3.1	Tube	3 mm	Beige	Convex	Positive	Negative
	GM 4.2	Tube	2 mm	Beige	Convex	Positive	Negative

These results indicate that the isolates are catalase negative. Based on the API 50 CHL test, it is known that generally lactic acid bacteria contained in all milk samples is Lactobacillus fermentum. Then Axelsson (2004) said that lactic acid bacteria are tube and round gram-positive bacteria and are negative catalase. Rizqiati et al. (2015) found lactic acid bacteria derived from buffalo milk present in North Sumatra, namely Lactobacillus plantarum, L. brevis, L. paracasei, L. pentosus and Lactococcus lactis. Surono (2016) stated that lactic acid bacteria is a bacterium that acts as a probiotic derived from milk and fermented milk product, which is beneficial for digestive tract health.

CONCLUSION

It can be concluded that the nutritional content of fresh milk obtained from cow, buffalo and goat in West Sumatra were already meet the requirements of fresh milk quality according to BSN No. 3141.1: 2011. Meanwhile, microbiologically all fresh milk samples had aerobic microbial contamination over the threshold allowed of $1x10^6$ CFU /ml. A high number of LABs indicated that milk in this study could potentially be a source of probiotics.

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Citation in the references:
Literatures in reference are written alphabetically based on the author's name. Same author is written sequentially starting from earlier order.

Example of reference writing

Primary paper:
Bhanja SK, Anjali DC, Panda AK, Sunder GS. 2009. Effect of post hatch feed deprivation on yolk-sac utilization and young broiler chickens. Asian-Aust J Anim Sci. 22:1174-1179.

Book:

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.

c. Paloheimo M, Piironen J, Vehmaanpera J. 2010. Xylanases and cellulases as feed additives. In: Bedford MR, Partridge GG, editors. Enzymes in farm animal nutrition. 2nd ed. New York (USA): CABI Publishing. p. 12-53.

Proceeding:

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.

Thesis:

Krisnan R. 2008. Kombinasi penggunaan probiotik mikroba rumen dengan suplemen katalitik pada pakan domba (Thesis). [Bogor (Indones)]: Institut Pertanian Bogor.

Electronic magazines:

Wina E, Tangendjaja B, Dumaria. 2008. Effect of *Calliandra calothyrsus* on *in vitro* digestibility of soybean meal and tofu wastes. Livest Res Rural Develop. Vol. 20 Issue 6. http://www.lrrd.org/lrrd20/6/wina 20098.htm.

Institution:

a. [NRC] National Research Council. 1985. Nutrient requirements of sheep. 6th revised. Washington DC (USA): National Academic Press.
b. [CDC] Centers for Disease Control. 2006. Standard operating procedure for the direct Rapid Immunohistochemistry Test (dRIT) for the detection of rabies virus antigen. [accessed December 20th 2011]. http://www.rabiesblue print.com/IMG/pdf/DRIT_SOP.pdf.

Patent:

Blanco EE, Meade JC, Richards WD. 1990. Ophthalmic ventures, assignee. Surgical stapling system. United States patent US 4,969,591. 1990 Nov 13.

10. **Citation in text:**

Citation consists author’s last name and publication year.

Example:

a. One author: .... grow slower than lamb fed cattle’s milk (Supriyati 2012). Supriyati (2012) formulates..........
b. Two authors: .... expect, end maintenance weight (Khasrad & Rusdimansyah 2012). Khasrad & Rusdimansyah (2012) argued..........
c. Three authors or more: .... based on DNA mitochondria analysis (Mtileni et al. 2011). Mtileni et al. (2011) reports.....
d. Same author cited from 2 different papers: (Purwadaria et al. 2003a, 2003b).
e. Author with same family name is written consecutive: (Dawson J 1986; Dawson M 1986).
f. Several different authors are written consecutive: (Kannan et al. 2000; Grandin 2007; Santosa et al. 2012).
g. Institution: CSA (2011)............

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