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VIABILITY ASSESSMENT OF FRESH CHICKEN BLASTODERMAL CELLS BY FLOW CYTOMETRY

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ABSTRACT

This study describes the viability assessment of fresh chicken blastodermal cells (BCs) by flow cytometry using a novel DNA-detecting far-red-fluorescing dye, DRAQ5 and LIVE/DEAD (LD) Fixable Green Dead Cell Stain Kit. Overall, the technique provides both quantitative and qualitative evaluation of BCs. Particular attention was put on the determination of a portion of nucleated ($DRAQ5^+$) and dead ($DRAQ5^+/LD^+$) BCs from freshly isolated samples. The results showed that the proportion of nucleated cells from a sample was ranged from 50.39 to 72.58 % and dead cells from 0.40 to 11.38 % in all repetitions. Furthermore, our study suggests that flow cytometry allows precise examination of fresh BCs viability in a very short time and that a combination of DRAQ5/LD staining is suitable for the detection of nuclear BCs and their viability evaluation, respectively. Moreover, this technique might also be applied for the evaluation of frozen-thawed BCs after cryopreservation process for animal gene bank.

Key words: chicken; blastodermal cells; viability; flow cytometry

INTRODUCTION

Chick embryos are an important model for developmental biology research and transgenesis. At stage X (Eyal-Giladi and Kochav, 1976), avian embryos already contain 40-60,000 BCs, and at this time the different tissues can be established. A small group of cells (less than 100), the primordial germ cells (PGCs), are clearly committed to form the germline (Carsience *et al.,*1993; Etches *et al.*, 1997; Kagami *et al.*, 1997; Pettite *et al.*, 1990; Thoraval *et al.*, 1994). These cells will be of the interest for germplasm preservation programs based on germline technologies, where germ cells of endangered breeds and species can be cryopreserved and later propagated through germline chimeras (Petitte, 2006).

Flow cytometry technique has been extensively used to investigate the cell viability. This technique is very popular for the analysis of cell suspensions containing live, apoptotic and necrotic cells because of their higher sensitivity (Liegler *et al.*, 1992), in comparison with other methods, such as Trypan blue assay. An accurate assessment of the cell viability necessitates the maintenance of the cell integrity and viability during the isolation procedures. DRAQ5 (1.5-bis {[2-(methylamino) ethyl] amino}-4, 8-dihydroxy anthracene-9,10-dione) is a specific dye with high DNA affinity which can be used to detect nucleated cells.

To determine viability of cells, „live-dead" kits are often used to detect necrotic cells. These dyes stain both viable and dead cells, since they specifically bind

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to the proteins. However, they stain dead cells much more brightly, making them easily distinguishable during analysis (Perfetto *et al.*, 2006).

The aim of our work was to assess the viability of freshly isolated BCs of Oravka breed using DRAQ5/LD staining by flow cytometry.

MATERIAL AND METHODS

Hatching eggs obtained from sexually mature (≥ 60 week old) and health ROSS 908 breeding hens (n = 10.000) reared in a private breeding facility (Liaharenský podnik Nitra a.s., Párovské Háje, Slovak Republic) were used in this experiment. Hens were maintained under artificial conditions (14 h of light at 10 lux and 10 h of dark) and were fed with a commercial diet (KV; TEKRO Nitra, s.r.o., Slovakia), and water was given *ad libitum*. The eggs were stored at 14-16 °C and 80 % of humidity for 4 days. The experiment with BCs samples was repeated six times (from February to May 2017).

To obtain blastoderms, each egg was broken and the yolk was separated from the albumen. The blastoderm was carefully covered with a filter paper ring (Petitte *et al.*, 1990). The yolk membrane was cut around the filter paper ring and blastoderm was washed several times in a calcium-magnesium free PBS (CMF-PBS, Applichem, Darmstadt, Germany) to remove excessive yolk. After cleaning, *area pellucida* was separated from *area opaca*, mechanically dispersed and single cells were stored for further analysis.

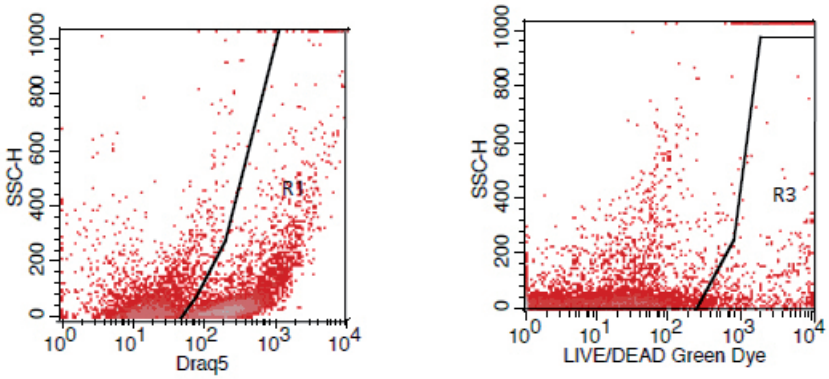

Fig. 1: Representative flow cytometry dot plots of fresh BCs. Region R1 (A) represents DRAQ5⁺ BCs (nucleated cells) and region R3 (B) represents DRAQ5⁺/LD⁺ dead (necrotic) BCs

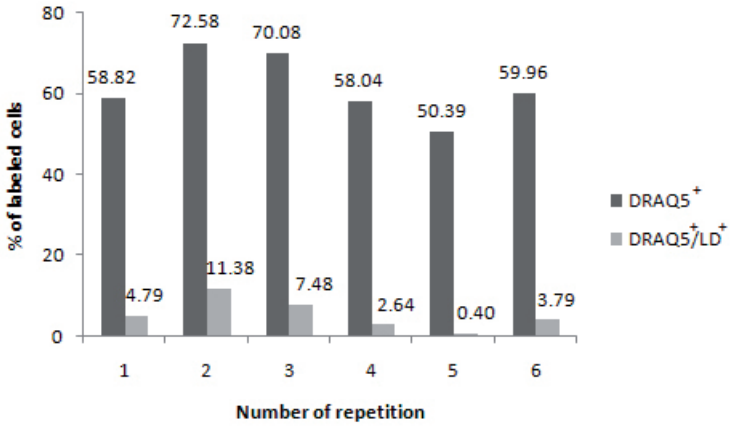

Fig. 2: The incidence of DRAQ5⁺ (nucleated) and DRAQ5⁺/LD⁺ (necrotic) BCs in cell suspension in different replications

The cell suspension was subdivided and placed into prepared tubes intended for flow cytometric assessment of nucleated and necrotic cells. Cells were stained with DRAQ5 (Biolegend, Germany) and LIVE/DEAD Fixable Green Dead Cell Stain Kit (Molecular Probes, Eugene, USA) according to the producer manual. DRAQ5 nuclear dye identifies the nucleated BCs, and the proportion of necrotic cells within the population was determined using the specific dye LIVE/DEAD Fixable Green Dead Cell Stain Kit (Figure 1). Samples were analysed by a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA).

RESULTS AND DISCUSSION

The different labelling patterns in the DRAQ5/LD analysis identify two different cell populations (Figure 2). The DRAQ5 nuclear dye was used for the determination of the nucleated cells in fresh samples. Using the LD dye, we identified two different cell subpopulations within nucleated cells, live ($DRAQ5^+/LD^-$) and necrotic ($DRAQ5^+/LD^+$).

Due to the high content of cellular debris in a freshly isolated cell suspension and different cell size it is very difficult to distinguish the BCs from other cells. However, Trypan blue method is widely used for detection of frozen-thawed BCs viability (Sawicka *et al.*, 2015; Chełmónska *et al.*, 1997; Thereshenko *et al.*, 1994), though it has certain limitations. The dye can be incorporated into live cells after a short exposure time, and personal reliability can affect the results (Avelar-Freitas *et al*., 2014). Thus, the viability of BCs cannot be properly discerned by the Trypan blue exclusion method. In our study, DRAQ5 dye was used to determine portion of nucleated cells and LD dye for the detection of dead BCs in freshly isolated samples. This allowed examination of the isolated cell population by a flow cytometry and characterization of cell viability through the necrosis-based pathways.

In previous study, flow cytometry protocol was also used to analyse viability of fresh chicken BCs (Hamidu *et al.*, 2010). In this study, viability of fresh BCs was evaluated basing on a propidium iodide staining and an annexin V assay.

CONSLUSION

In conclusion, we showed in this work that flow cytometry could become useful technique for evaluation of BCs quality. DRAQ5 staining allows determination of nucleated cells from the cell suspension of freshly isolated sample, and in combination with LD kit also dead cells can be detected. These findings might also be applied to the field of cryopreservation to determine the frozen-thawed cell viability.

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COMPARISON OF RABBIT ENDOTHELIAL PROGENITOR CELLS AND MESENCHYMAL STEM CELLS: CYTOGENETIC APPROACH

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ABSTRACT

The aim of this preliminary work was to analyze metaphases of endothelial progenitor cells isolated from peripheral blood and mesenchymal stem cells harvested from rabbit bone marrow in order to assess the effect of culture conditions on the karyotype. We analyzed 30 metaphases of both endothelial cells and mesenchymal stem cells of a rabbit. Our preliminary results show that 73.3 % of endothelial cells retained stable number of chromosomes, while in mesenchymal stem cells we recorded moderately lower percentage (66.6 %). Changes in the genome may lead to changes in the gene expression as well as to dysfunctions of the cell activity. Therefore, it is necessary to analyze chromosomal abnormalities in order to monitor culture conditions, particularly in cells intended for the therapeutic use.

Key words: rabbit; endothelial progenitor cells; mesenchymal stem cells; karyotype

INTRODUCTION

Except the endothelial cells that form the vasculature, non-hematologic endothelial progenitor cells (EPCs) are also present in the bloodstream (Asahara *et al.*, 1997; Yin *et al.*, 1997; Shi *et al.*, 1998; Gehling *et al.*, 2000; Lin *et al.*, 2000). Originating from adult bone marrow tissue, EPCs have the similar features as embryonal angioblasts. These precursor cells have the high capacity to proliferate and to differentiate into mature ECs (Hristov and Weber 2003; Urbich and Dimmeler 2004).

Mesenchymal stem cells (MSCs) are present in many adult tissues. The first tissue where the MSCs were identified was bone marrow (BM-MSCs). Stem cells with this kind of origin possess various advantages in comparison to MSCs of other tissue derivates: high osteogenic differentiation capacity, well investigated properties already applied in use with biomaterials, not ethically controversial background (Kang *et al.*, 2012). MSCs are also capable of high proliferation and multi-lineage differentiation (Jin *et al.*, 2013). However, invasivity of the BM-MSCs harvesting initiates the interest in finding more accessible sources of MSCs (Pontikoglou *et al.*, 2011).

In opposite to the embryonic stem cells, in which chromosomal disorder has been widely reported, MSCs are characterized as genetically stable during culture (Borgonovo *et al.*, 2014). However, karyotype changes in both endothelial and mesenchymal stem cells are associated with donor age and their incidence increases after the fifth passage. Previous research has shown that abnormalities in chromosomal count may result in carcinogenesis (Campos *et al.*, 2009; Miyai *et al.*, 2008). Due to tumorogenic threats it is recommended to investigate the chromosomal count after *in vitro* culture. For the purpose of monitoring of chromosomal stability, Moralli *et al.* (2011) suggested the use of microarray-based techniques: Comparative Genomic Hybridization (CGH), single nucleotide polymorphism (SNP) analysis and transcriptional profiling. Nevertheless, from the perspective of clinical routine, the classical cytogenetic protocols, such as G-banding, are the less cost-consuming but sufficient for objective analysis. Therefore, our study

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aimed to analyze metaphases of the endothelial progenitor cells and stem cells isolated from rabbit bone marrow in order to assess the effect of culture conditions on the cell karyotype.

MATERIAL AND METHODS

Biological material

Clinically healthy rabbits of New Zealand White line were used in the experiment. Rabbits were cage held in a partially air-conditioned hall of a rabbit farm at the NPPC Research Institute for Animal Production Nitra, Slovakia. Rabbits were humanely sacrificed and peripheral blood and femoral bones were processed afterwards.

Isolation of endothelial cells

Peripheral blood was collected into the prepared tubes with anticoagulant (EDTA). Rabbit mononuclear cells of the peripheral blood (PBMCs) were isolated using Biocoll (Biochrom, Berlin, Germany) density-gradient centrifugation. Thereafter, cells were resuspended in an EBM-2 basal medium (Lonza, MD, USA) supplemented with recombinant growth factors (kit; EGM-2 SingleQuots™; CC-4176, Lonza), 20 % of fetal bovine serum (FBS) and antibiotics (1 % of penicillin and streptomycin) (Life Technologies). Cells were immediately placed onto T75 tissue culture flasks and cultured until passage 3 (P3), as described by Vašíček *et al*. (2016a).

Isolation of mesenchymal stem cells

Mononuclear cells were isolated using Biocoll solution from the rabbit bone marrow. Centrifuged cells were resuspended in a MEM-Alpha medium supplemented with FBS and antibiotics and then plated into T75 tissue culture flasks. Cells were cultured for 3 weeks until the P3, as previously described by Vašíček *et al*. (2016b).

Karyotype analysis

Samples for chromosome analysis were processed as follows. After passaging, actively growing cells from P3 were incubated with KaryoMAX® Colcemid™ solution in HBSS (Hanks' Balanced Salt Solution,) (Life Technologies, Slovak Republic) for 16 h at 37 °C and 5 % CO_2. Treated cells were washed with Dulbecco's phosphate buffered saline (DPBS; Gibco BRL, USA) and dissociated by 0.05 % trypsin (ThermoFisher, Slovak Republic). After centrifugation (200 g for 10 min), cells were resuspended and incubated in a hypotonic solution (75 mM KCl) for 20 min at 37 °C. Following centrifugation, harvested cells were incubated with 5 ml of chilled fixative consisting of methanol and acetic acid (3:1; v/v) for 10 minutes. Chromosome spreads were prepared by dropping the cell suspension onto pre-chilled glass slides. The air-dried cell spreads were stained by 2 % Giemsa solution (Gibco BRL, USA) and observed under a light microscope at 400 x magnification. A normal rabbit karyotype consists of 22 pairs (2n = 44) of chromosomes. Chromosomal abnormality was defined as following: hyperploidy - > 44, hypoploidy - < 44. The final percentage of abnormal karyotype was defined as (< 2n) + (> 2n).

RESULTS AND DISCUSSION

The samples were divided into two groups according to the cell type. Metaphase of BM-MSCs is shown in Figure 1 and of EPC in Figure 2.

Our preliminary results show that for endothelial cells the percentage of diploid cells was 73.3 %. An abnormal karyotype occurred in 26.6 %, of which 10 % were hyperploid and 16.6 % hypoploid cells. Miyai *et al.* (2008), who harvested corneal endothelial cells from human cadavers, reported that increased aneuploidy occurs not only in older donors but also in later cultures and passages. Our results correspond to this hypothesis of incidence of chromosomal abnormalities along with increased number of passages.

Concerning MSCs, the proportion of diploid cells was lower (66.6 %) in comparison to EPCs, however chi-square test showed no significant differences between EPCs and MSCs. Abnormal karyotype was observed in 33.5 % of cells, of which 10 % were hyperploid and 23.5 % hypoploid. This phenomenon could be due to the fact that MSCs grew slower than EPCs during culture.

Table 1: Karyotype analysis of endothelial progenitor cells and mesenchymal stem cells from bone marrow

Type of cells	Total number of cells (N)	2n % / N	< 2n % / N	> 2n % / N	Total % of abnormal karyotypes
Endothelial progenitor cells	30	73.3 / 22	16.6 / 5	10 / 3	26.6
Mesenchymal stem cells	30	66.6 / 20	23.5 / 7	10 / 3	33.5

N = number of cells; 2n = diploidy (normal); < 2n = aneuploidy: hypoloidy; > 2n = aneuploidy: hyperploidy

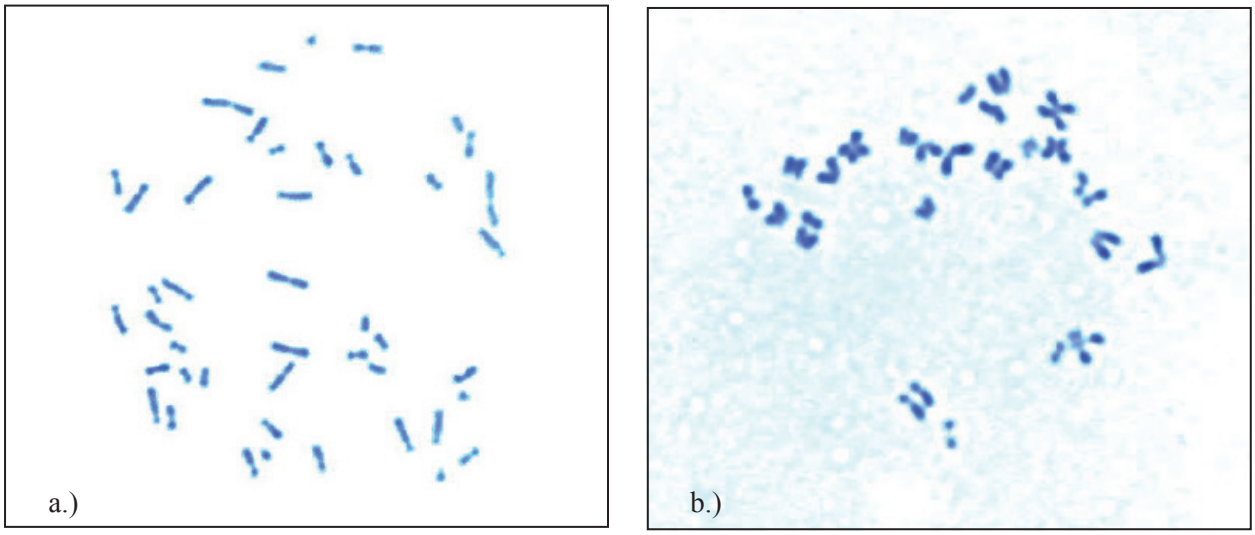

Fig. 1: Bone marrow-derived mesenchymal stem cells: a, normal number b, decreased number – hypoploidy

This outcome does not corresponds with the study of Asadi-Yousefabad *et al.* (2015), who claimed that growing passaging of canine BM-MSCs does not affect the karyotype.

These preliminary findings show that chromosome malformations arise during culture. Observation of karyotypes from the first passage until at least the fifth passage could provide more precise results. Nevertheless, chromosomal aberrations in cells intended for future therapeutic use are a real concern due to association with carcinogenesis (Campos et al., 2009). Therefore, it is recommended to thoroughly evaluate the karyotypes of the cell colonies before differentiation or clinical application.

CONCLUSION

Our preliminary results confirm the importance of cytogenetic study, since this technique is able to detect numeric chromosomal aberrations. Chromosomal abnormalities were detected in both rabbit endothelial progenitor cells and mesenchymal stem cells derived from the bone marrow.

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INCIDENCE OF DAIRY COW MASTITIS AND ASSOCIATED RISK FACTORS IN SODO TOWN AND ITS SURROUNDINGS, WOLAITA ZONE, ETHIOPIA

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ABSTRACT

A longitudinal observational study on the incidence of mastitis in smallholder dairy cows in Sodo town and its surroundings, Ethiopia was conducted during the period from October 2015 to March 2016 with the aim of estimating incidence of mastitis in smallholder dairy cows, investigating potential risk factors associated with mastitis, and isolating mastitis-causing bacteria in milk of smallholder dairy cows. All the sixty-seven lactating cows from the nine smallholder dairy farms were registered for the follow up study. The incidence of mastitis at cow and quarter levels was followed up for six months. On top of that, a questionnaire survey on smallholder dairy cow management and milking procedure was performed at the farms where the study animals resided. The results of this study revealed 50.7 % (n = 34/67) prevalence of mastitis at cow and 29.1 % (n = 78/234) at quarter levels. The total incidence risk was found to be 60.6 % (n = 20/33). Based on bacteriological examination, 90.8 % of the collected samples (n = 59/65) were found to be mastitis bacteria positive. Pathogenic bacteria belonging to five genera were involved in causing mastitis. Among these isolates, *Staphylococcus aureus* was the predominant mastitis pathogen (n = 23/65, 39 %) found in the study area followed by *Streptococci* species (n = 12/65, 20.3 %), Coagulase-negative *Staphylococci* species (n = 11/65, 18.6 %), *Escherichia coli* (n = 8/65, 13.6 %), and *Bacillus* species (n = 3/65, 5.0 %). The least mastitis pathogen isolated was *Corynebacterium* spp. (n = 2/65, 3.4 %). The influence of 22 potential risk factors on the incidence of mastitis was also investigated. Among others, late lactation stage, low daily milk yield, male milkers, dry cow therapy only at last milking of lactation were elicited to be highly risky for mastitis. On the other hand, milkers' work experience, herd size, teat distance from the ground, and milkers' skill of finding milk clots appeared to be not risky for the incidence of mastitis whereas, age greater than or equal to eight year, parity above or equal to six, and milk yield less than three liters per day were significantly associated with the prevalence of mastitis ($p < 0.01$. $p < 0.05$, $p < 0.05$, respectively). Although not significant, owners as milkers and late lactation stage had higher influence on the prevalence of mastitis ($p = 0.058$ and $p = 0.147$, respectively). In conclusion, the relatively high incidence of mastitis in the study area can be responsible for serious impact on the economy of smallholder dairy farmers mainly by reducing the quantity and quality of milk yield and undermining fertility of the dairy cows. Thus, continuous education of the smallholder dairy farmers is needed for better mastitis control programs.

Key words: mastitis; dairy cows; risk factors; udder pathogen

INTRODUCTION

Causative agents of mastitis with zoonotic potential may represent a health risk for human populations via food chain (Bradley, 2002). Thus, extra attention should be paid to the study of mastitis.

Mastitis, inflammation of the parenchyma of mammary gland is a complex disease of dairy cows (Idriss *et al*., 2013). It is accompanied by physical, chemical, pathological and bacteriological changes in milk and glandular tissue (Samad, 2008). Almost any bacterial or mycotic organism that can opportunistically invade tissue and cause infection can cause mastitis. Over 135 different microorganisms have been isolated from bovine intra-mammary infections, but the majority of infections are caused by staphylococci, streptococci, and gram-negative

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bacteria (Bradley, 2002). Mastitis of dairy cows is often described as subclinical or clinical mastitis. Subclinical mastitis is the presence of an infection without apparent signs of local inflammation or systemic involvement. It is always related to low milk production, changes to milk consistency, reduced possibility of adequate milk processing, low protein and high risk for milk hygiene since it may even contain pathogenic organisms (Tancin *et al.*, 2007; Sharma *et al.*, 2011). Whilst what the farmer sees is clinical mastitis, subclinical mastitis is more serious and is responsible for much greater loss to the dairy industry (Kader *et al*., 2002). Cows with subclinical mastitis should be considered a risk of spreading mastitis pathogens within and between herds and are as such of a national concern (Person *et al*., 2011). It is, therefore, important to know the prevalence of subclinical mastitis in dairy herds and delineate the important factors responsible for it.

Detection of subclinical mastitis is best done by examination of milk for somatic cell counts using either the California Mastitis Test (CMT) or automated methods. The CMT is the most reliable and inexpensive cow side test for the detection of subclinical mastitis worldwide. It is an indirect measure of cell count. The CMT reagent contains a detergent that reacts with DNA of the cell nuclei, and a pH indicator (Bromo cresol purple) that changes the color when the milk pH increases above its normal value (Radostits *et al.*, 2007). Somatic cells are mainly milk-secreting epithelial cells that have been shed from the lining of the gland and white blood cells (mainly neutrophyls) that have entered the mammary gland in response to injury or infection. They are normal constituent of milk and only when they become excessive do they indicate intra-mammary infection (Reksen *et al*., 2008). Somatic cells are composed of leukocytes (75 %) and epithelial cells (25 %) (Henna Hamadani *et al.*, 2013). The leukocytes are attracted to the area of inflammation, where they attempt to fight the infection. In general, it is accepted that somatic cell count (SCC) is a golden standard in diagnostics of any form of mastitis in udder (Pyörälä, 2003).

In Sodo town and its surrounding, the number of smallholder dairy farms has a tendency to increase. However, the economic benefit they acquire from dairy farms is not inspiring. Although it is assumed that mastitis plays important role in the reduction of milk quantity and quality and poor fertility of dairy cows in the study area, limited studies have been conducted on the contribution of mastitis, especially subclinical mastitis, to the problem of these smallholder dairy herds and on their management practices related to mastitis problems. The study on the incidence of mastitis, mastitis causative bacteria, and factors associated with mastitis is thus important to design relevant mastitis prevention strategy. Therefore, this study was carried out with the objectives of estimating incidence of mastitis in smallholder dairy cows in Sodo town and its suburb, investigating factors associated with the incidence of mastitis, and isolating bacteria causing mastitis in milk of smallholder dairy cows.

MATERIAL AND METHODS

Study animals

A longitudinal observational study was conducted during the period from October 2015 to March 2016 to estimate incidence of mastitis in smallholder dairy cows. All the sixty-seven lactating cows that were found in nine smallholder dairy farms were registered for the follow up study. The study animals were managed under intensive farming system. The housing system was tie-stall. They were milked by hand twice a day at the tie stall. The animals were provided either with green grass or hay and/or crop residue according to the availability of the feed. They were supplemented with wheat bran. The animals were visited by investigators every month during the morning milking time for six consecutive months (on days 0, 30, 60, 90, 120, 150, and 180). During each visit, their mammary glands were first visually then physically examined for any pathological change and for the presence of blind teats.

Milk samples also collected from these dairy cows were immediately subjected to physical examination with naked eyes to detect any abnormalities in color, odor, consistency and presence of clot, blood, flakes and any other visible abnormalities. In addition to the physical examination of the milk, CMTs were performed at the farms to determine whether the dairy cow is positive or negative for mastitis. California Mastitis Test (CMT; Bovi-VetTM, Kruuse, Germany) was carried out on all quarters with the exception of quarters with blind teats. The milk samples were collected, after the quarters were washed and dried, the first few squirts of milk were discarded and about 3 ml of milk was collected from each quarter into the respective wells of the CMT paddle and an equal volume of CMT reagent was added into each well. The paddle was swirled to thoroughly mix the contents for about 20 seconds. According to the amount of gel formed, the reaction was visually scored according to the formation of reaction or not as “negative” and “positive”. On day zero visit, mastitis positive dairy cows were identified and excluded from the follow up study. The remaining animals were considered as mastitis at risk and followed up for the incidence of mastitis. Thereafter, at each visit of the smallholder dairy farms, mastitis positive cows were excluded from the follow up study. Further, management practices, housing conditions, and milking routine of each small holder dairy farm were observed during each visit. All examinations and data collection for this study were carried out by the investigators.

Milk sample collection for bacteriological isolation

The milk samples were taken shortly prior to milking and only cows with strongly CMT positive quarters were sampled. A total of 65 quarters from the nine smallholder farms were sampled for bacteriological isolation. The milk samples were collected by a standard milk sampling technique as described by Quinn *et al.* (2002). After a quarter had been cleaned up by removing any possible dirt and washed with water, the teat end was dried and swabbed with cotton soaked in 70 % ethyl alcohol. Approximately 10 ml of milk was collected aseptically into sterile bottles, after discarding the first three milking streams. Milk samples from each quarter were transported to Wolaita Zone Regional Laboratory in an ice-cooled box at 4 ºC and analyzed immediately for identification of the mastitis pathogen.

Media preparation

Both general purpose and selective media were prepared and used according to their manufacturers' guidelines. Measured amount of media was added to a flask containing known volume of distilled water and placed into hot plate stirrer until it boils. Then, bacteriological media were placed within the autoclave and sterilized at 121 ºC for 15 minutes. The medium was cooled to 45–50 ºC inside water bath and 5 % sterile sheep blood was added to blood agar base medium for the growth of fastidious *Streptococcus* species that require enriched media. Then, the media was poured into different size petri-plates under aseptic condition inside Bio-safety Cabinet (Bioair Instruments, Eurolone Company, Italy) and allowed some time to solidify. Similarly, the agar media was also poured into test tube and slants were made. On the other hand, broth media were prepared in a test tube inside bio-safety cabinet. After preparation, all bacteriological media were placed into an incubator adjusted to 37 ºC for overnight to check the growth of contaminant. Petri-plates and test tubes free from contaminant were used for culturing of milk sample.

Identification of bacteria

A loopful of each milk sample was inoculated on blood agar base enriched with 5 % defibrinated sheep blood (Oxoid Ltd, Basingstoke, Hampshire, England) (Wasilauskas *et al.*, 1974); and incubated for 18–24 hours at 37 °C. Then returned to the incubator for at least another 24–48 hours and reexamined for the presence of slow growing bacteria. Different colonies were sub-cultured and incubated again on blood agar base and MacConkey agar (Oxoid Ltd, Basingstoke, Hampshire, England) until clear separated colonies were observed in a petri-plate. Then, the pure colonies were transferred to nutrient agar (Oxoid Ltd, Basingstoke, Hampshire, England) and allowed to grow inside the incubator. The identification of bacteria were made using colony morphology, hemolytic characteristic; gram staining, catalase test, coagulase test, CAMP test and IMViC (Indole, Methyl red, Voges-Proskauer, Citrate) (Cheesbrough, 2006; Quinn *et al.*, 2002). Additionally, the isolation of microbes were made using selective and differential media like MacConkey agar, Bacillus cereus agar, Mannitol salt agar and purple base agar (Oxoid Ltd, Basingstoke, Hampshire, England), and Eosin methylene blue agar (Himedia, Mumbai, India).

Questionnaire survey

Data from each animal and herd were collected using an individual questionnaire. The purpose of the questionnaire survey was to gather information on the farm and its management practices in addition to each visit observation of the farm. Accordingly, parameters studied were age, breed, number of parity, lactation stage and per day milk production, milking procedure, milkers' experience and their sex, manure management in the farm. Age, parity, lactation stage were determined by asking owner and farm attendant as well as from the farm records where available.

Statistical analysis

The collected data were checked for any inconformity and inconsistence and entered into Excel spreadsheet, coded, and transferred to SPSS version 20. Both the questionnaire and CMT data were analyzed on the given statistical package software. For descriptive statistics presentation of categorical data, Chi-square was used to compare the different groups of age, sex, and various risk factors, with the outcome variable (mastitis). A p-value less than 0.05 was considered statistically significant.

RESULTS

A total of 67 lactating dairy cows from nine smallholder dairy farms were examined for the presence of mastitis. Of these, 34 dairy cows (50.7 %) were found to be mastitis positive. All the milk samples collected from 234 quarters were subjected to CMTs. Seventy eight (29.1 %) of them showed positive reaction for mastitis. The prevalence of mastitis at cow level was highest in herds where small numbers of cows were lactating compared to herds with high number of lactating cows (Table 1). Twenty nine point four percent (n = 10) of the 34 mastitis positive dairy cows had one quarter infected, while 26.5 % (n = 9) had two quarters, 29.4 % (n = 10) three quarters, and 14.7 % (n = 5) four quarters affected (Table 2). The majority of blind teats originated from a farm where Jersey breed cows were reared (28.6 %, n = 32/112 quarters). In general, a total of 34

Table 1: Prevalence of mastitis at cow and quarter levels

Farm	No. of cows examined	No. of CMT positive cows	Cow level prevalence (%)	No. of quarters examined	CMT positive quarters	Quarter level prevalence (%)
F01	9	5	55.6	36	14	38.9
F02	9	5	55.6	36	16	44.4
F03	28	10	35.7	112	14	12.5
F04	3	1	33.3	12	2	16.7
F05	3	3	100.0	12	6	50.0
F06	3	3	100.0	12	6	50.0
F07	3	1	33.3	12	2	16.7
F08	4	3	75.0	16	10	62.5
F09	5	3	60.0	20	8	40.0
Total	67	34	50.7	268	78	29.1

Table 2: The proportion of quarter affected from mastitis positive cows

Farm	No. of quarter affected and prevalence									
	one	%	two	%	three	%	four	%	total (+) cows	%
F01	1	10.0	0	0.0	3	30.0	1	20.0	5	14.7
F02	0	0.0	1	11.1	2	20.0	2	40.0	5	14.7
F03	7	70.0	2	22.2	1	10.0	0	0.0	10	29.4
F04	0	0.0	1	11.1	0	0.0	0	0.0	1	2.9
F05	1	10.0	1	11.1	1	10.0	0	0.0	3	8.8
F06	1	10.0	1	11.1	1	10.0	0	0.0	3	8.8
F07	0	0.0	1	11.1	0	0.0	0	0.0	1	2.9
F08	0	0.0	1	11.1	0	0.0	2	40.0	3	8.8
F09	0	0.0	1	11.1	2	10.0	0	0.0	3	8.8
Total	10	29.4	9	26.5	10	29.4	5	14.7	34	100.0

Table 3: The prevalence of blind teat in different farms

Farm	No. of quarter examined	No. of blind teat	Prevalence (%)
F01	36	0	0.0
F02	36	2	5.6
F03	112	32	28.6
F04	12	0	0.0
F05	12	0	0.0
F06	12	0	0.0
F07	12	0	0.0
F08	16	0	0.0
F09	20	0	0.0
Total	268	34	12.7

Table 4: The incidence risk of mastitis in the study area

Farm	No. of animal initially at risk	No. of animal affected in the study period	Incidence risk (%)
F01	4	2	50.0
F02	4	4	100.0
F03	18	11	61.1
F04	2	1	50.0
F05	0	-	-
F06	0	-	-
F07	2	1	50.0
F08	1	1	100.0
F09	2	0	0.0
Total	33	20	60.6

Table 5: The incidence and relative risks of mastitis in association with animal risk factors

Risk factors		No. of animal initially at risk	No. of animals affected in the study period	Incidence risk (IR) (%)	Relative risk (RR)
Age (years)	3–5	16	10	62.5	1.1
	5–8	10	5	50.0	1.4
	≥ 8	7	5	71.4	
Parity	Primiparous	13	7	53.8	1.4
	2–5	16	10	62.5	1.2
	≥ 6	4	3	75.0	
Stage of lactation (months)	1–4	14	6	42.9	1.7
	4–7	3	2	66.7	1.1
	≥ 7	16	12	75.0	
Breed	Jersey	19	12	63.2	
	HF	12	7	58.3	1.1
	Cross	2	1	50.0	1.3
Milk yield (Lts)	< 3	8	8	100.0	
	3–8	16	9	56.3	1.8
	> 8	9	3	33.3	3.0
Teat distance from the ground	> 50 cm	15	9	60.0	
	< 50 cm	18	11	61.1	1.0

HF = Holstein Friesian

out of 268 quarters (12.7 %) were found to be blind in this study (Table 3).

The highest mastitis incidence risk was found in dairy farms, F02 and F08, which was 100 % (n = 4/4, n = 1/1, respectively) followed by F03 showing 61.1 % (n = 11/18) incidence risk of mastitis. Three of the remaining farms, F01, F04, and F07 had 50 % (n = 2/4, n = 1/2, n = 1/2, respectively). The total incidence risk of mastitis in the study area during the study period was found to be 60.6 % (n = 20/33) (Table 4).

Age group greater than eight years was 1.4 times more at risk for the incidence of mastitis than age group five to eight years and 1.1 times more at risk than age group three to five years. Animals on parity greater

than six were at higher risk to be affected by mastitis (1.4 times than primiparous), followed by dairy cows on two to five parity (1.2 times) and primiparous cows. Dairy cows at late stage of lactation were at higher risk of being affected by mastitis than animals at early and mid-lactation stages (1.7 times, 1.1 times, respectively). Jersey dairy cows were more at risk of mastitis, followed by Holstein Friesian, and Cross breeds (Exotic x Local) (Jersey dairy cows are 1.3 times at risk for mastitis than the cross breeds and 1.1 times than the Holstein Friesian cows). In this study, lowest milk production was found to be three times more at risk for mastitis than daily milk yield greater than eight liters and one point eight times than three to eight liter milk yield per day (Table 5). Management factors such as work experience of milkers, skill of finding milk clots in the milk streaks, and herd size were not associated with mastitis. Dairy farms that are secondary means of income for their owners are 1.3 times more at risk for mastitis than dairy farms that are the primary means of income. In addition, dairy cows that are hand-milked by male milkers are 1.5 times more at risk of acquiring mastitis than cows milked by female hand milkers. Dry cow therapy and removal of manure only once in a day were found to be 100 % risky for mastitis (Table 6). Washing udder and teat with flowing water was found to be 1.8 times more risky for mastitis than washing by soaking hand in a bucket of water. Examination of first streaks of milk was 1.3 times riskier for mastitis than not examining. Using common towel for drying udder before milking was found to be greater risk factor (1.5 times) for mastitis than using a new towel for each cow. In this study, milking dried udder and drying milker's hands before milking were shown to be higher risk factors for mastitis (1.2 times, 1.5 times, respectively) than milking wet udder and milking with wet hands of the milker. Similarly, washing hands after milking each cow was shown 1.2 times riskier for mastitis than not washing hands. Pre and Post-milking teat dipping were not practiced in the smallholder dairy farms (Table 7).

Prevalence of mastitis in dairy cows was

Table 6: The incidence and relative risks of mastitis in association with management

Variable	Description	No. of farms	No. of animal initially at risk	No. of animal affected	IR (%)	RR
As source of income	Primary	3	22	12	54.5	
	Secondary	6	11	8	72.7	1.3
Milker‘s sex	Male	4	26	17	65.4	1.5
	Female	5	7	3	42.9	
Experience of milker	≤ 5 years	3	5	3	60.0	
	> 5 years	6	28	17	60.7	1.0
Skill of identifying	Yes	6	27	17	63.0	1.3
sick udder	No	3	6	3	50.0	
Skill of finding milk clots	Yes	4	25	15	60.0	
Feeding cows	No	5	8	5	62.5	1.0
just after milking	Yes	4	10	7	70.0	1.2
Dry cow therapy	No	5	23	13	56.5	
	Yes	1	4	4	100.0	1.8
Regular surveillance	No	8	29	16	55.2	
of dry udder	Yes	2	6	5	83.3	1.5
Manure removal	No	7	27	15	55.6	
from the stall	Once/day	2	4	4	100.0	1.5
	> Once/day	7	29	16	55.2	
Herd size	≤ 10 animals	5	5	3	60.0	
	> 10 animals	4	28	17	60.7	1.0

IR = incidence risk, RR = relative risk

significantly ($p < 0.01$) associated with adult age of animals compared to young adult and young age (80.8 % vs 50.0 % vs. 17.4 %, respectively). Significantly more dairy cows ($p < 0.05$) at higher parity were found to be affected by mastitis than cows at 2–5 parity and primiparous (69.6 % vs 53.8 % vs 22.2 %, respectively). Low milk yield per day significantly ($p < 0.05$) influenced the prevalence of mastitis compared to relatively high and medium yielding dairy cows (83.3 % vs 52.0 % vs 36.7 %, respectively). Teat distance from the ground and lactation stages were not significantly ($p = 0.542$ and $p = 0.147$, respectively) associated with the prevalence

Table 7: The incidence and relative risks of mastitis in association with milking procedure

Variable	Description	No. of farms	No. of animal initially at risk	No. of animal affected	IR (%)	RR
Udder and teat washed by	Soaking hand in water	8	29	16	55.2	
	Flowing water	1	4	4	100.0	1.8
Milking with	Wet udder	2	4	2	50.0	
	Dry udder	7	29	18	62.1	1.2
Drying towel	Common	4	22	15	68.2	1.5
	Individual	5	11	5	45.5	
First streaks examination	Yes	4	27	17	63.0	1.3
	No	5	6	3	50.0	
Washing hands after milking each cow	Yes	3	10	7	70.0	1.2
	No	6	23	13	56.5	
Drying hands before milking	Yes	3	24	16	66.7	1.5
	No	6	9	4	44.4	

Table 8: Association of animal factors with prevalence of mastitis

Variable	Description	Mastitis prevalence	Total no. of animals	X^2	df	p-value
Age	3–5	4 (17.4 %)	23	19.6182	2	0.000
	5–8	9 (50.0 %)	18			
	≥ 8	21 (80.8 %)	26			
Parity	Primiparous	4 (22.2 %)	18	9.218	2	0.010
	2–5	14 (53.8 %)	26			
	≥ 6	16 (69.6 %)	23			
Lactation stage (months)	< 4	6 (33.3 %)	18	3.841	2	0.147
	4–7	6 (46.2 %)	13			
	≥ 7	22 (61.1 %)	36			
Milk yield	< 3	10 (83.3 %)	12	7.493	2	0.024
	3–8	11 (36.7 %)	30			
	≥ 8	13 (52.0 %)	25			
Teat distance from the ground (cm)	< 50	23 (76.7 %)	30	0.537	1.0	0.542
	≥ 50	31 (83.8 %)	37			

X^2 = Chi-square, df = degree of freedom, Significance = $p < 0.05$

of mastitis in dairy cows kept in smallholder farms. However, percentage of prevalence of mastitis was elevated with increased lactation stage compared to medium and early lactation stages (61.1 % vs 46.2 % vs 33.3 %, respectively) (Table 8). The highest prevalence of mastitis (p = 0.058) was found in lactating dairy cows milked by owners of the farm compared with cows milked by employee (100 % vs. 75.9 %). More dairy cows were affected by mastitis (p = 0.236) in smallholder dairy farms where the farm is used as secondary source of income than cows kept in farms that are used as primary source of income (87.5 % vs. 75 %). The percentage of dairy cows having mastitis was also higher (p = 0.342) in cows which were milked by different milkers every other day than in cows milked by the same milker (83.4 %, 73.1 %, respectively). Similarly, although not significant, increased prevalence of mastitis occurs in cows, which were milked by high school educated workers than in cows milked by primary school educated workers (87.1 %, 75 %, respectively). Further, results of this study revealed that prevalence of mastitis in cows was not influenced by milkers' work experience and frequency of manure removal (p = 0.514, p = 1.00, respectively) (Table 9).

The results of bacteriological culture on the milk samples collected from mastitis dairy cows considering only strong CMT positive samples are indicated in Table 10. 90.8 % of the collected samples (n = 59/65 samples)

Table 9: Association of farm management practices with prevalence of mastitis

Variable	Description (no. of farm)	Mastitis prevalence (%)	Total no. of animals	X^2	df	Significance
Farm as source of income	Primary (3)	27 (75.0)	36			
	Secondary (6)	27 (87.5)	31	1.559	1.0	0.236
Milker's work status	Employee (5)	41 (75.9)	54			
	Owner (4)	13 (100)	13	3.883	1.0	0.058
A cow is milked by	The same milker (4)	19 (73.1)	26			
	Different milker (5)	35 (83.4)	41	1.536	1.0	0.342
Milker's work experience	> 5 (7)	43 (79.6)	54			
	≤ 5 (2)	11 (84.6)	13	0.167	1.0	0.514
Milker's educational status	High school (6)	27 (87.1)	31	1.559	1.0	0.236
	Primary (3)	27 (75.0)	36			
Frequency of manure removal	Once/day	10 (83.3)	12	0.070	1.0	1.000
	> Once/day	44 (80.0)	55			

X^2 = Chi-square, df = degree of freedom, Significance = $p < 0.05$

Table 10: Bacteria isolated from mastitis dairy cows

Bacterial isolate	Frequency of isolation	Percentage of isolation (%)
Saphylococcus. aureus	23	39.0 %
Coagulase–Negative *Staphylococcus* spp	11	18.6 %
Streptococcus species	12	20.3 %
Bacillus species	3	5.0 %
E. coli	8	13.6 %
Corynaebacterium species	2	3.4 %
Total	59	90.8 %
No growth	6	9.2 %

were found to be mastitis bacteria positive whereas 9.2 % of the examined samples were negative (n = 6/65). Bacteria belonging to five genera were involved in causing mastitis. Both contagious and environmental bacteria were isolated. *Staphylococcus aureus* was the predominant mastitis pathogen (39 %, n = 23/65) found in the study area followed by *Streptococcus* species (20.3 %, n = 12/65), Coagulase–negative *Staphylococcus* species (18.6 %, n = 11/65), *Escherichia coli* (13.6 %, n = 8/65), and *Bacillus* species (5.0 %, n = 3/65). The least mastitis pathogen isolated was *Corynebacterium* spp. (3.4 %, 2/65).

DISCUSSION

The prevalence of mastitis at cow level in this study (50.7 %, n = 54/67) was almost similar to the findings (53.30 %) of Rahman *et al.* (2010) in Bangladesh, Bradley *et al.* (2007) (47.0 %) in Great Britain, Hashemi *et al.* (2011) (44.7 %) in Iran. It was higher than the results reported by Belayneh *et al.* (2013) (39.5 %) in Central Ethiopia, Girma *et al*. (2012) (23.18 %) in Eastern part of Ethiopia, and Abdel-Rady and Mohammed Sayed (2009) (19.14 %) in Egypt. On the other hand, it was less than the prevalence recorded by Yien Deng *et al.* (2015) (60.33 %) in Western Ethiopia and Muhamed Mubarak *et al*. (2012) (66.0 %) in India. The prevalence of dairy cows' mastitis in the Central and Eastern part of the country was lower than the prevalence in this study area (Southern part of the country) probably because the studies in those areas were conducted on cross and native breed dairy cows, respectively. Native and cross breed dairy cows are more resistant to mastitis compared to exotic breeds, which constituted above 90 % of the study animals in this study. The quarter level prevalence of mastitis (29.4 %) in the study area was comparable with the finding of Belayneh *et al.* (2013) and Hashemi *et al.* (2011) (23.7 % and 21.6 %, respectively) while it was much lower than the prevalence reported by Person *et al.* (2011), Muhamed Mubarak *et al*. (2012), Idriss *et al*. (2013), and Yien Deng *et al.* (2015) (60.0 %, 66.0 %, 73.85 %, and 47.21 %, respectively). This study revealed that 12.7 % of the quarters were blind (n = 34/268), which was a lot higher than the results reported by Girma *et al*. (2012) and Yien Deng *et al.* (2015) (2.2 %, n = 34/384 and 0.21 %, n = 1/484, respectively). Most of the blind teats (94.1 %) occurred in Jersey breed dairy cows (F03). This might be due to relatively wider teat openings and shorter teat distance from the ground; the teats are highly exposed to injury and thereby to blindness. Another finding of this study, which claimed that the Jersey breed dairy cows were 1.3 times more likely to develop mastitis than cross breed cows and 1.1 times more at risk than Holstein Friesian cows (Table 5) supports the above result.

According to the results of our study, the risks and prevalence of mastitis increased with advancing age, parity and lactation stage. Eight year and older dairy cows were 1.4 times at risk for mastitis compared to young adults (5–8 years old) and 1.1 time more at risk than young cows (3–5 years old). Similarly, dairy cows in parity number six and above were 1.4 times more at risk for mastitis than primiparous and 1.2 times more at risk than cows in parity number two to five. Correspondingly, Chi-square analyses revealed that prevalence of mastitis was significantly higher in adult (≥ 8 year old), cows at greater or equal to six parity, and cows yielded less than three liters of milk per day ($p < 0.01$, $p < 0.05$, and $p < 0.05$, respectively). These observations support the results recorded by Kerro and Tareke (2003), Islam *et al.* (2010), Girma *et al.* (2012), and Tancin (2013). It is possible to postulate that older cows have increased susceptibility due to depressed host defense mechanism. Age of cows approximates with parity. High risk of acquiring mastitis and prevalence of mastitis ($p = 0.147$) in dairy cows occurred in late lactation stage probably because of inefficient immune system response due to gradual change of feed formulation to dry cow diet, and stress triggered by advancing gestation. In this study, the greatest risk of developing mastitis and significantly higher ($p < 0.05$) prevalence of mastitis happened in daily low milk yielder cows (< 3 liters of milk.day^{-1}). Milk yield decreases as the lactation progresses. This coincided with the result reported by Du Preez (2000) where the somatic cell count usually increased only after the milk production of the cow failed to less than 4 kg per day. According to Radostits *et al.* (2007), prevalence of mastitis increases as the stage of lactation progresses. Research also showed that cows milked intermittently towards the end of lactation have dramatically increased somatic cell count (Blowey and Edmondson, 2000). This finding, therefore, asserted the aforementioned finding of the study; "there is high risk of acquiring mastitis in late lactation stage". Teat distance from the ground, milkers' skill of physical examination of milk, milkers' work experience, and herd size did not influence the incidence of mastitis in smallholder dairy farms in this study.

Smallholder dairy farms which were secondary means of income for the owners were more at risk for mastitis than farms that were primary source of income. Similarly, prevalence of mastitis was almost significantly high ($p = 0.058$) in smallholder dairy farms, where owners milk dairy cows (Table 9). 88 % of the surveyed smallholder dairy farms served as supplementary source of income to the owners. The smallholder dairy farmers operated with a very small resource base and earned much less than they required for livelihood from the farm. Thus, they cannot employ workers for the farm work.

In addition to milking and caring for thr animals, they have to spend much of their time working in other places to fill the gap in their costs of living. This probably partially diverted their attention that should have been fully paid to mastitis control and prevention. Dairy cows that were hand-milked by male milkers were highly (1.5 times more) at risk of acquiring mastitis compared to cows hand-milked by female workers. This might be because a woman knows better how to take care of dairy cows and handle proper hygienic conditions in the milking process. In this study, feeding cows just after milking and dry cow therapy just after the last milking of lactation were unexpectedly found to be highly risky for incidence of mastitis than cows not fed after milking and not treated during the early part of the dry period. Short acting antibiotics (Quick-release antibiotics) were used for dry cow therapy in the study area (Personal observation). Thus, the antibiotics remained active for a short period of time and probably protected the udder health in early dry period and thereafter intra-mammary infection might have occurred during the remaining dry period. This is supported by pioneer finding of Smith *et al.* (1985) where dry cow therapy was not effective during the pre-partum period. In addition, observational studies have shown that most infections with coliform and environmental streptococci take place in the last two weeks before calving (https://ahdc.vet.cornell.edu/programs/NYSCHAP/docs/). Peterson-Wolfe *et al.* (2010) also confirmed that cows are most susceptible to mastitis pathogens in the last seven to ten days of the dry period. Further, dry-cow therapy should be applied in conjunction with other mastitis control measures. Therefore, early dry and pre-partum periods can generally be considered critical for udder health. Based on this, it is safe to infer that in order to make dry cow therapy successfully effective in preventing udder infections to minimize the incidence of mastitis and ensure a production of safe for consumption milk, early dry period therapy should be repeated at two weeks pre-partum.

According to Jones (2006) and Idriss *et al.* (2013), the teat canals may remain partially open for 1–2 hour after milking. Hence, feeding cows just after milking is important to make animals remain standing to prevent pathogens from freely entering through the open teat canal. However, in this study a result opposite to the expected was documented. The explanation to this finding could be that the animals might be offered neither quality nor adequate amount of feed that could make them stand for 1–2 hours. Further, udder and teat washing with flowing water, milking with dry udder, first strips examination, washing hands after milking each cow and drying hands before milking unexpectedly failed to positively influence incidence of mastitis in the studied smallholder dairy farms. Most of the smallholder dairy farms in the study area were using unsanitized hand-borewell or river water for any purpose in the farms. They were also using common cloth towels for drying cows (Table 7) According to Peterson-Wolfe (2010), water should not be used as part of any milking procedure even if a sanitizing solution is added. According to him, sanitizers do not maintain activity throughout a milking, and water can introduce pathogens that are very difficult to cure. Using unsanitized hand-borewell or river water in the milking procedure probably had detrimental effect on unexpected results obtained in this study regarding to the incidence of mastitis. On top of using the unsanitized water, milkers' hand washing and udder washing were not carried out in accordance to valid norms. During udder washing, teat ends around the orifice were usually overlooked. The remaining dirt around the teat orifice might harbor mastitis-causing pathogens, which might freely enter into the teat canal during milking and cause intra-mammary infection. The milker's hands were simply rinsed with water only. Forestripping sub-clinically infected dairy cows and not properly washing hands might have served as means of transmission of mastitis to uninfected cows in the study area. Henna Hamadani *et al.* (2013) declared that the milker's hands should be washed thoroughly with disinfecting soap before milking. To further to prevent mastitis, Jones (2006) suggested approaching the milking procedure in the same way a surgeon approaches surgery: wash hands with soap and water, wash teats and udder in sanitizing solution, thoroughly dry teats and udder with individual towels, dip teats in an effective germicidal teat dip. Moreover, using common cloth towel to dry wet udder and teats of different cows might spread pathogens from sick/reservoir animal to other cows in the studied herds. As Henna Hamadani *et al.* (2013) reported, mastitis pathogens spread rapidly from cow to cow in the absence of pre and post-milking teat dipping. In these animals, transmission of mastitis infections can also occur through flies, especially by *Hydrotaea irritans* (Vasil, 2009).

90.8 % of the collected samples (n = 59/65 samples) were found to be mastitis bacteria positive. Whereas, 9.2 % (n = 6/65) of the strongly CMT positive collected milk samples were found to be mastitis bacteria negative. Bacteria-negative samples may occur due to spontaneous bacterial cure, the presence of too few viable bacteria for culture techniques, or death of the bacteria after removal of the milk sample from the gland but prior to culture (Zorah *et al*., 1993). Of the isolated bacteria, *Staphylococcus aureus* was the predominant mastitis pathogen (n = 23/59, 39 %) found in the study area followed by *Streptococcus* species (n = 12/59, 20.3 %), Coagulase–Negative *Staphylococcus* species (n = 11/59, 18.6 %), *Escherichia coli* (n = 8/59, 13.6 %), and *Bacillus* species (n = 3/59, 5.1 %). The least mastitis pathogen isolated was *Corynebacterium* spp. (n = 2/59, 3.4 %). Similarly, *Staphylococcus aureus* was the principal pathogen in Czech Republic, Denmark, and Germany

(Rysanek *et al.*, 2007; Schwarz *et al.*, 2010; Mohammed *et al.*, 2013). The prevalence of *E.coli* and *Bacillus* spp. in this study was close to the findings of Idriss *et al.* (2013) (12.3 % and 6.41 %, respectively), whereas the prevalence of *Staphylococcus aureus* in this study was much higher than claimed by Idriss *et al.* (2013) (9.74 % vs. 39 %). Among the bacterial culture isolates of this study, *Staphylococcus aureus* and *E.coli* belong to the most important major pathogens involved in bovine mastitis worldwide (Olde Riekerink *et al.*, 2008). *Staphylococcus aureus* is considered contagious (Barkema *et al.*, 1998) but environment *Staphylococcus aureus* mastitis may also occur (Zadoks *et al.*, 2002). *E. coli* is mainly of environmental origin (Munoz *et al.*, 2007). Other pathogens have both routes of infection (Idriss *et al.*, 2013). According to Sumathi *et al*. (2008), the relatively high incidence of environmental mastitis was due to poor hygiene of housing and milking conditions, as environment pathogens infect the udder through teat canal.

CONCLUSION

Incidence of mastitis at cow and quarter levels was found to be relatively high in the study area and can have serious economic impact on smallholder dairy farmers by reducing the quantity and quality of milk and undermining fertility of the dairy cows. The association of 22 potential risk factors with dairy cow mastitis was investigated. Of these factors, adult age, late lactation stage, low daily milk yield, male milkers, dry cow therapy only at last milking of lactation were elicited to be highly risky for mastitis. Similarly, adult age of dairy cows, increasing parity, and lower milk yield were significantly associated with the prevalence of mastitis. Using unsanitized hand-borewell or river water in the milking procedure, not washing hands and udder of dairy cows in accordance to valid norms, and using common cloth towel for drying udder and teats had detrimental effect on the incidence of relatively high mastitis. In order to minimize the incidence of mastitis, dry-cow therapy should be applied both at early dry period and at two weeks pre-partum in conjunction with other mastitis control measures.

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PHENOTYPIC CORRELATIONS BETWEEN THE EGG WEIGHT, SHAPE OF EGG, SHELL THICKNESS, WEIGHT LOSS AND HATCHLING WEIGHT OF TURKEYS

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ABSTRACT

Eggs of turkey layers from the North Caucasian bronze (NCB) breed were investigated. Incubation eggs were monitored on days 9 and 15 to establish embryonic mortality rates. By the 25th day of incubation, before moving the eggs in the incubator, they were weighed to determine the weight loss by this moment. Thirty eggs, from which normal turkey poults have hatched, were randomly selected. The weight and thickness of eggshells were determined, the hatchlings were weighed and their relative weight from the egg was calculated. A moderate positive correlation was established between the weight of incubated hatched eggs of turkeys at 34 and 46 weeks of age and eggshells weight ($r = 0.51$ and $r = 0.50$, respectively). Eggshell thickness was proportional to the whole egg weight – heavier eggs had thicker shells ($r = 0.34$; $p < 0.05$). A substantial positive effect of egg weight on the hatchling weight was established ($r = 0.77$; $r = 0.65$ at $p < 0.001$). The observed correlations between the shape of eggs and turkey egg weight, shell weight, shell thickness of hatched eggs, weight loss during incubation, were low and negative. A positive correlation ($r = 0.52$) was demonstrated between the weight and thickness of eggshells in eggs produced by 34-week-old and from 46-week-old turkeys ($r = 0.68$; $p < 0.001$). A negative correlation (to $r = -0.80$) existed between weight loss during incubation and the absolute and relative weight of the hatchling.

Key words: turkey eggs; correlation; shape index; shell weight; shell thickness; loss of weight; hatchling turkeys weight

INTRODUCTION

Efficient selection in poultry farming depends on the knowledge about the relationships between the different productive traits. It is acknowledged that the different parameters characterizing egg quality influence the quality of hatched chickens.

Different authors (Brunson and Godfrey, 1953; Wilson, 1991; Narushin's and Romanov, 2002) have demonstrated the effect of egg weight and eggshell thickness on the egg weight loss during the incubation and the size of the hatchling.

The weight of hatchlings usually ranges between 62-76 % of egg weight and correlates strongly with it (Wilson and Harms, 1988; Bondarenko, 1989; Halaj and Veterani, 1998). The minimum proportions of hatchlings from incubated eggs recommended by Islam *et al*. (2008) are 64-66 %.

In an experiment with turkeys, Reinhart and Moran (1979) reported that hatchlings comprised 60-67 % of incubated egg weight. In our previous studies on turkeys, the ratio between hatchling weight and incubation egg weight was from 65.59 % to 72.19 % (Hristakieva *et al.*, 2008). Shanawany (1987) has calculated that the hatchling weight increased by 0.59 g for each 1 g increase in egg weight.

Numerous authors (Pinchasov, 1991; Wilson, 1991; Shashina, 1995; Khurshid *et al*. 2003; Abiola *et al.* 2008; Egbeyale *et al*. 2011) reported a positive relationship between the weight of egg set and the weight of hatchlings. The relative weight at hatch is largely dependent on the egg weight. In Japanese quails, Alkan *et al.* (2008) established a strong positive correlation between both traits ($r = 0.72$). Yamak *et al.* (2015), also reported a strong correlation ($r = 0.862$) between the egg

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weight and hatchling weight at $p < 0.01$.

Egg shape index is defined as the ratio between its width and its length. The importance of this parameter consists in the role of egg shape in the direction of turning during incubation and determination of embryo movements for nutrients utilization.

Previous studies (Ozcelik, 2002; Nowaczewski *et al.*, 2008; Yakubu *et al*., 2008 Kgwatalala *et al*. 2016) reported a weak correlation between the weight of eggs and egg shape index. Bernacki and Heller (2003) found strong positive correlation between the egg shape index and egg weight, suggesting that heavier egg were more rounded. The observed correlations between turkey egg shape index and egg weight were low and negative (Hristakieva, 2010).

According to Suk and Park (2001) and Ozcelik (2002), there was a positive relationship between the thickness and weight of his eggshell with whole egg weight. Harms *et al*. (1990) demonstrated that the eggshell weight and thickness was proportional (0.92-0.97) to the size of the whole egg. Both Zhang *et al*. (2005) and Wolanski *et al*. (2007) provided data about a strong correlation between eggshell weight and thickness. Sahan *et al*. (2003) found out a negative relationship ($r = -0.65$) between the eggshell thickness and the incubation egg weight loss in ostrich eggs. They reported the strongest weight loss of 13.03 % in eggs with the thinnest shells, while the respective values in shells of moderate and great thickness were 11.22 % and 10.36 %.

The aim of this research was to establish the relationships between the weight and the shape of incubation eggs, the weight and thickness of the eggshells, the weight loss during incubation and the weight of the hatchling in turkeys at 34- and 46-weeks of age.

MATERIAL AND METHODS

The study was carried out in the breeding turkey farm of the Selection, population genetics, reproduction and technologies of poultry and rabbits research department at the Agricultural Institute – Stara Zagora in 2016.

Eggs from turkey layers from the North Caucasian Bronze (NCB) breed were investigated. The birds were reared at the breeder farm on deep permanent litter at a population density of 3 birds.m^{-2}. They were fed a standard ration containing metabolizable energy – 12.55 MJ.kg^{-1}, crude protein – 18.10 %, calcium – 2.87 %, available phosphorus – 0.49 %. Average daily feed intake was 300 g. The turkey layers were artificially inseminated twice a week with 0.025 ml fresh semen, undiluted.

Incubation of eggs was done in "Optima" incubators. Every incubation egg was numbered and weighed. Monitoring for embryonic death was performed on days 9 and 15. By the 25th day of incubation, eggs were weighed before being moved to the incubator to determine incubation weight loss by the equation:

Egg weight loss during incubation to 25 d of incubation (%) = $(EW_1-EW_2)/EW_1*100$,

Where: EW_1- egg weight prior to the incubation;

EW_2- egg weight by the 25th day of incubation before moving to the hatch

Eggs were placed in special egg plates with partitions to determine the individual hatch of each of the poults. Randomly, 30 eggs from which normal viable birds have hatched, were selected. The eggshell weight and thickness were determined, hatchlings were weighed and their relative weight from the whole egg weight was calculated.

Table 1: Weight and shape of incubation eggs, eggshell weight and thickness, weight loss during incubation and hatchling weights for eggs of 34- and 46-week-old turkey layers (mean ± SD)

Parameters	Eggs of 34-week -old turkeys		Eggs of 46-week -old turkeys	
	x ± SD	Coefficient of variation (%)	x ± SD	Coefficient of variation (%)
Egg weight(g)	82.04 ± 0.760	5.10	84.22 ± 0.780	4.99
Shape index (%)	74.25 ± 0.490	3.65	71.57 ± 0.600	4.54
Shell weight (g)	6.73 ± 0.100	8.02	7.24 ± 0.080	6.21
Shell thickness (mm)	0.39 ± 0.005	5.13	0.38 ± 0.005	5.26
Egg weight loss during incubation at 25 d of incubation (%)	9.96 ± 0.250**	13.86	12.47 ± 0.420	17.96
Chicks weight (g)	55.93 ± 0.780	7.63	54.45 ± 0.740	7.34
Chicks weight (%)	68.16 ± 0.620*	4.94	64.59 ± 0.660	5.48

*$p < 0.05$; ** $p < 0.01$

The statistical analysis of data was performed with Excel 2003- ANOVA using the following statistical tests: Descriptive Statistics, t-Test: two-Sample Assuming Equal Variances, Correlation. The statisticall significance of correlation coefficients was determined using the Students'T criterion and the formula: $S_{rp} = 1\text{-}r_p^2/\sqrt{n\text{-}2}$; $T = r_p/S_{rp}$, where: r_p - correlation, S_{rp}- standart error of correlation (Zhelyazkov and Tsvetanova, 2002).

RESULTS AND DISCUSSION

Table 1 presents the average values of incubation eggs' weight and shape, the eggshell weight and thickness, weight loss during incubation and hatchlings' weight for turkeys aged 34 and 46 weeks. There were no statistically significant differences between turkeys at both studied ages with respect to egg weight and shape, shell weight and thickness, and hatchling weights. Higher percentage of weight loss during incubation – 12.47 % was exhibited by turkeys at 46 weeks of age compared to 9.96 % for eggs laid by younger turkeys. Hence, the relative hatchling weight was by 3.57 % lower in the eggs of 46-week-old birds. Robel (1981) and Soipes (1992) also reported differences in weight loss during incubation related to the age of layers. Coefficients of variation of egg weight and shape, shell weight and thickness for turkeys at 34 and 46 weeks of age were comparable to those reported by Oblakova (2005) for turkey eggs and by Kul and Seker (2004) in Japanese quail eggs. The highest coefficient of variation was observed for the egg weight loss during 25-day incubation – 17.96 % for eggs of 46-week-old birds and 13.86 % for eggs of 36-week--old layers.

Table 2 presents the calculated correlations between weight and shape of incubation eggs, eggshell weight and thickness, weight loss during incubation and hatchling weights for eggs of 34- and 46-week-old turkey layers. For both studied ages, the weight of incubation eggs correlated weakly and positively (r = 0.11 and r = 0.13)

Table 2: Correlations between weight and egg parameters

Parameters	Egg weight (g)	Shape index (%)	Shell weight (g)	Shell thickness (mm)	Egg weight loss to 25 d of incubation (%)	Chicks weight (g)	Chicks weight (%)
Egg weight (g)							
eggs of 34-week -old turkeys	-	0.13	0.51***	0.34*	-0.31	0.77***	0.14
eggs of 46-week -old turkeys	-	0.11	0.50***	0.34*	0.01	0.65***	-0.04
Shape index (%)							
eggs of 34-week -old turkeys	-	-	-0.14	-0.20	0.14	-0.01	0.12
eggs of 46-week -old turkeys	-	-	0.31	0.39*	-0.04	0.07	-0.08
Shell weight (g)							
eggs of 34-week -old turkeys	-	-	-	0.52***	0.38*	0.28	-0.09
eggs of 46-week -old turkeys	-	-	-	0.68***	0.16	0.17	-0.20
Shell thickness (mm)							
eggs of 34-week -old turkeys	-	-	-	-	-0.23	0.23	0.002
eggs of 46-week -old turkeys	-	-	-	-	-0.15	0.09	-0.17
Egg weight loss to 25 d (%)							
eggs of 34-week -old turkeys	-	-	-	-	-	-0.45***	-0.36
eggs of 46-week -old turkeys	-	-	-	-	-	-0.59***	-0.80***
Chicks weight (g)							
eggs of 34-week -old turkeys	-	-	-	-	-	-	0.74***
eggs of 46-week -old turkeys	-	-	-	-	-	-	0.73***
Chicks weight (%)							
eggs of 34-week -old turkeys	-	-	-	-	-	-	-
eggs of 46-week -old turkeys	-	-	-	-	-	-	-

*p < 0.05; ***p < 0.01

to egg shape. This is in line with our previous studies on the correlation between shape index and weight of eggs from three studied lines of turkeys (Hristakieva, 2010). The correlation between egg weight and shell weight was moderate to strong ($r = 0.50$ and $r = 0.51$) at $p < 0.001$. The investigations of Choi *et al.* (1983) and Oblakova (2006) provide evidence that the eggshell weight was positively influenced by the weight of the whole egg. The observed correlation coefficients among the other studied traits showed that the weight of incubation eggs and hatchling weight exhibited the strongest correlation $r = 0.65$ and $r = 0.77$ respectively, at $p < 0.001$. These results are comparable with those of other researchers (Abiola *et al*., 2008; Alkan *et al*., 2008; Egbeyale *et al*., 2011; Yamak *et al*., 2015).

A highly significant positive correlation as found out between the weight and thickness of investigated eggshells from layers at 34 weeks of age ($r = 0.52$ vs $r = 0.68$) in eggs from turkeys at 46 weeks of age. This is important for determination of eggshell strength in breeder eggs.

It should be noted that the weight loss during incubation (by the 25th day) correlated negatively (up to $r = -0.80$) with both absolute and relative hatchling weights, hence the lower the egg weight loss, the higher the hatchling weight.

A negative relationship was observed between egg weight loss during incubation and eggshell thickness in younger turkeys: $r = -0.23$ as compared to $r = -0.15$ in older birds. In ostrich eggs, these traits also correlated negatively (Sahan *et al.*, 2003).

CONCLUSION

Positive correlation was established between the weight of incubated hatched eggs of turkeys at 34 and 46 weeks of age and eggshells weight ($r = 0.51$ and $r = 0.50$, respectively). Eggshells thickness was proportional to the whole egg weight – heavier eggs had thicker shells ($r = 0.34$; $p < 0.05$). Substantial positive effect of egg weight on the hatchling weight was established ($r = 0.77$; $r = 0.65$ at $p < 0.001$). The observed correlations between the shape and weight of eggs, shell weight, shell thickness of hatched eggs and weight loss during incubation were low and negative. Positive correlation ($r = 0.52$) was demonstrated between the weight and thickness of eggshells in egg produced by 34 –week--old and 46-week- -old turkeys ($r = 0.68$; $p < 0.001$). Negative correlation (up to $r = -0.80$) existed between weight loss during incubation and absolute and relative weight of the hatchling.

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EVALUATION OF EFFECTS OF A STRAIN, STOCKING DENSITY AND AGE ON BILATERAL SYMMETRY OF BROILER CHICKENS

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ABSTRACT

The study was conducted to assess bilateral symmetry of broiler chicken strains raised on different stocking densities. The study lasted for eight weeks with 216 broiler chickens belonging to three different strains used for the experiment: Marshal, Ross 308 and Arbor Acres. The birds were randomly allotted to three different stocking densities of 10, 12 and 14 birds.m^{-2}, respectively. Each treatment was replicated twice in a 2 ×3 factorial design. Left and right leg length, wing length and face length of the same birds from each group were measured weekly from 35th to 56th days of age using a digital caliper. The morphological data collected on the birds were used to estimate bilateral symmetries. Data were subjected to analysis of variance (ANOVA) using a Statistical Analysis System, SAS (2004). The results showed that effect of strains on bilateral symmetry of face length of the broiler chickens was not significant ($p > 0.05$). The bilateral symmetry of the wing length was significantly affected by a strain of the broiler chickens. The results showed that effect of stocking density on bilateral symmetry of face length of the broiler chicken was not significant. The bilateral symmetry of wing length was significantly affected by the stocking density of the chicken. There was a significant effect of age on bilateral symmetry of face length of the chicken. There were significant interaction effects of strain and stocking density on bilateral symmetry of broiler chickens. Based on results of the study it is concluded that the bilateral symmetry of morphological traits, as estimated by directional asymmetry, fluctuating asymmetry and relative asymmetry, were affected by a strain of the chicken and stocking density. Thus, the strain and stocking density may be claimed as important factors affecting developmental stability of broiler chickens.

Key words: bilateral density; stocking; strain; symmetry

INTRODUCTION

Broiler chickens (*Gallus gallus domesticus*) are a gallinaceous domesticated fowl bred raised specifically for meat production. They are usually raised as mixed--sex flocks in large sheds under intensive condition, but some strains can be raised as free-range flock. Most commercial broilers reach slaughter weight at five to seven weeks of age, although slower growing strains reach slaughter weight at approximately 14 weeks of age (Kruchten, 2002). There are different strains of broilers which include Arbor Acres, Anak 2000, Marshal, Ross and Hubbard. Arbor Acres, Marshal and Ross were among the strains of broiler chicken reared by farmers in Nigeria. They cope fairly well with the hot season of January to March in Nigeria and reach market weight at about 8 weeks of age (Udeh and Ogbu, 2011). In broiler production, a stocking density (floor space per chicken) is very important welfare factor, minimal standard in relation to welfare of broilers are focused on space for their walking, which is the main prerequisite for development of locomotive apparatus and demonstration of basic forms of behavior (Skubic *et al.*, 2007).

Fast growing broiler chickens have a large appetite and high body weight gain in short time. This large appetite and some other environmental conditions together may cause problems such as flip-over syndrome, left-right side differences of bilateral traits and some other health problems (Mendes *et al*., 2007). The situation where the right and left sides of the animals are not grown at equal levels or do not display a similar growth are seen often. This is a situation which depends on genetic structures,

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rearing and the other environmental conditions of animals (Campo *et al*., 2005). Bilateral symmetry, the deviation of a part of an organism from perfect symmetry can be categorized as anti-symmetry, directional, or fluctuating (Van Valen, 1962; Mirtagioglu *et al*., 2013). It is an undeniable fact that the performances of symmetric animals are higher than those which are not symmetric (Manning and Ockenden, 1994). Thus, this study sought to assess the bilateral symmetry of broiler chicken raised at different stocking densities.

MATERIAL AND METHODS

The study was conducted in a commercial poultry farm in Ibadan, Nigeria. The dimension of each pen was 2.01 m^2 (2.01 m x 1.00 m) and it was constructed in a way as to permit straight-through ventilation. The birds were fed commercial broiler feed ration (2900 kcal.kg ME^{-1} and 20.00 % crude protein). Fresh feed and clean water were supplied *ad libitum*. The feeders and drinkers were served proportionally depending on the number of birds per treatment. Vaccination schedule and other management practices were strictly kept. The study lasted for eight weeks. Totally 216 day-old broiler chicks, consisting of three different strains, were used for the experiment: Marshal, Ross 308 and Arbor Acres. The birds were randomly allotted to three different stocking densities: 10, 12 and 14 birds.m^{-2} respectively. Each treatment was replicated twice in a 2 ×3 factorial design.

Data collection

Left and right leg length, wing length and face length of the same birds from each group were measured weekly from 35th to 56th days of age (after breeding the chicks for four weeks) using a digital caliper. The morphological data collected on the birds were used to estimate bilateral symmetries as early described (Yalcin *et al*., 2003; Mendes, 2008; Mirtagioglu *et al*., 2013). Directional asymmetry (DA), fluctuating asymmetry (FA), anti-symmetry (AS) and relative asymmetry (RA) were used as measures for deviation from bilateral symmetry. DA was defined as mean not zero with normal distribution. Therefore, DA is an asymmetry in which growth on a given side consistently exceeds that on the other side. AS was defined as mean zero with non-normal distribution and FA was defined as mean zero with normal distribution based on absolute differences between left and right sides (L-R). FA is one of the measures of developmental instability of quantitative properties resulting from errors in developmental processes. Therefore, the FA can be defined as the asymmetry due to chance fluctuation in the development of the left and right sides of body. RA was defined as the ratio of the absolute value of left-right differences divided by the value for the size of the trait.

Statistical analysis

Data were subjected to analysis of variance (ANOVA) using statistical analysis System, SAS (2004), where statistical differences occurred, the means were separated using Duncan Multiple Range Test (DMRT).

Table 1: Effects of strains on bilateral symmetry of broiler chickens (mean ± SD) (mm)

Strains	Bilateral symmetry	Face length	Wing length	Leg length
Arbor Acres	Fluctuating asymmetry	0.12 ± 0.02	0.37 ± 0.05^{ab}	0.04 ± 0.03
	Anti – Symmetry (FA)	0.12 ± 0.02	0.37 ± 0.50^{ab}	0.04 ± 0.03
	Relative asymmetry	3.65 ± 0.65	2.10 ± 0.29^{ab}	0.02 ± 0.01
	Directional asymmetry	3.40 ± 0.03	17.73 ± 0.10^{b}	272.48 ± .34^{b}
Marshal	Fluctuating asymmetry	0.12 ± 0.02	0.28 ± 0.05^{b}	0.06 ± 0.02
	Anti – Symmetry (FA)	0.12 ± 0.02	0.28 ± 0.05^{b}	0.06 ± 0.02
	Relative asymmetry	3.41 ± 0.48	1.50 ± 0.30^{b}	0.02 ± 0.01
	Directional asymmetry	3.37 ± 0.03	18.13 ± 010^{a}	276.56 ± 2.73^{ab}
Ross	Fluctuating asymmetry	0.13 ± 0.01	0.48 ± 0.04^{a}	0.02 ± 0.03
	Anti – Symmetry (FA)	0.13 ± 0.01	0.48 ± 0.04^{a}	0.02 ± 0.03
	Relative asymmetry	3.78 ± 0.44	2.71 ± 0.24^{a}	0.01 ± 0.01
	Directional asymmetry	3.38 ± 0.02	17.85 ± 0.11^{ab}	283.43 ± 200^{a}

$^{a, b}$ means of different superscripts along the same column are significantly different ($p < 0.05$); SD – Standard deviation

RESULTS AND DISCUSSION

Table 1 shows the effect of strains on bilateral symmetry of the broiler chickens. The results showed that effect of strains on bilateral symmetry of face length of the broiler chickens was not significant ($p > 0.05$). The bilateral symmetry of the wing length was significantly affected by the strain of the broiler chicken ($p < 0.05$). Ross chicken had the highest FA for wing length. This implies that the Ross strain has large deviation from perfect symmetry (bilaterally asymmetrical). Palmer and Strobeck (1992) reported that FA was the highly

Table 2: Effects of stocking densities on bilateral symmetry of broiler chicken (mean ± SD) (mm)

Stocking densities	Bilateral symmetry	Face length	Wing length	Leg length
10 bird.m^{-2}	Fluctuating asymmetry	0.12 ± 0.02	0.47 ± 0.04^{a}	0.03 ± 0.04
	Anti – Symmetry (FA)	0.12 ± 0.02	0.47 ± 0.04^{a}	0.03 ± 0.04
	Relative asymmetry	3.26 ± 0.47	2.63 ± 0.24^{a}	0.01 ± 0.02
	Directional asymmetry	3.39 ± 0.03	18.08 ± 0.9^{a}	274.96 ± 2.66^{b}
12 bird.m^{-2}	Fluctuating asymmetry	0.11 ± 0.02	0.27 ± 0.05^{b}	0.03 ± 0.02
	Anti – Symmetry (FA)	0.11 ± 0.02	0.27 ± 0.05^{b}	0.03 ± 0.02
	Relative asymmetry	3.42 ± 0.62	1.49 ± 0.28^{b}	0.01 ± 0.01
	Directional asymmetry	3.36 ± 0.02	17.96 ± 0.11^{b}	281.11 ± 2.32^{a}
14 bird.m^{-2}	Fluctuating asymmetry	0.11 ± 0.02	0.38 ± 0.05^{ab}	0.05 ± 0.01
	Anti – Symmetry (FA)	0.11 ± 0.02	0.38 ± 0.05^{ab}	0.05 ± 0.11
	Relative asymmetry	3.34 ± 0.57	2.19 ± 0.30^{ab}	0.02 ± 0.01
	Directional asymmetry	3.40 ± 0.03	17.87 ± 0.11^{b}	276.42 ± 3.24^{b}

$^{a, b}$ means of different superscripts along the same column are significantly different ($p < 0.05$); SD – Standard deviation

Table 3: Effects of age on bilateral symmetry of broiler chicken (mean ± SD) (mm)

Age (days)	Bilateral symmetry	Face length	Wing length	Leg length
28	Fluctuating asymmetry	0.12 ± 0.03^{b}	0.42 ± 0.05	0.09 ± 0.02
	Anti – Symmetry (FA)	0.12 ± 0.03^{b}	0.42 ± 0.05	0.09 ± 0.02
	Relative asymmetry	3.99 ± 0.86^{b}	2.47 ± 0.30^{b}	0.01 ± 0.01
	Directional asymmetry	3.16 ± 0.02^{b}	16.84 ± 0.07^{b}	275.56 ± 2.98^{b}
35	Fluctuating asymmetry	0.12 ± 0.01^{b}	0.42 ± 0.06	0.09 ± 0.03
	Anti – Symmetry (FA)	0.12 ± 0.01^{b}	0.42 ± 0.06	0.09 ± 0.03
	Relative asymmetry	4.07 ± 0.38^{ab}	2.83 ± 0.33^{b}	0.01 ± 0.01
	Directional asymmetry	3.45 ± 0.02^{b}	18.09 ± 0.07^{ab}	276.21 ± 2.67^{b}
42	Fluctuating asymmetry	0.14 ± 0.01^{a}	0.42 ± 0.05	0.11 ± 0.06
	Anti – Symmetry (FA)	0.14 ± 0.01^{a}	0.42 ± 0.05	0.11 ± 0.06
	Relative asymmetry	4.16 ± 0.25^{a}	3.17 ± 0.24^{a}	0.02 ± 0.02
	Directional asymmetry	3.59 ± 0.01^{b}	19.06 ± 0.05^{a}	278.69 ± 3.80^{a}
49	Fluctuating asymmetry	0.15 ± 0.01^{a}	0.45 ± 0.05	0.15 ± 0.01
	Anti – Symmetry (FA)	0.15 ± 0.01^{a}	0.45 ± 0.05	0.15 ± 0.01
	Relative asymmetry	4.47 ± 0.01^{a}	3.86 ± 0.30^{a}	0.03 ± 0.01
	Directional asymmetry	4.29 ± 0.02^{a}	19.51 ± 0.12^{a}	278.71 ± 3.80^{a}

$^{a, b}$ means of different superscripts along the same column are significantly different ($p < 0.05$); SD – Standard deviation

Table 4: Interaction effects of strain × stocking density on bilateral symmetry of broiler chickens

Variables (mm)	Arbor Acres			Marshal			Ross		
	10 birds.m^{-2}	12 birds.m^{-2}	14 birds.m^{-2}	10 birds.m^{-2}	12 birds.m^{-2}	14 birds.m^{-2}	10 birds.m^{-2}	12 birds.m^{-2}	14 birds.m^{-2}
FAFL	0.04 ± 0.03^{b}	0.04 ± 0.04^{b}	0.07 ± 0.04^{a}	0.03 ± 0.03^{b}	0.01 ± 0.02^{c}	0.05 ± 0.03^{b}	0.02 ± 0.02^{c}	0.02 ± 0.03^{c}	0.06 ± 0.02^{a}
ASFL	0.04 ± 0.03^{b}	0.04 ± 0.04^{b}	0.07 ± 0.04^{a}	0.03 ± 0.03^{b}	0.01 ± 0.02^{c}	0.05 ± 0.03^{b}	0.02 ± 0.02^{c}	0.02 ± 0.03^{c}	0.06 ± 0.02^{a}
RAFL	1.14 ± 0.81^{c}	4.47 ± 1.22^{b}	5.34 ± 1.23^{a}	0.86 ± 0.86^{d}	0.41 ± 0.74^{d}	0.09 ± 0.03^{e}	3.50 ± 0.61^{b}	6.19 ± 0.93^{a}	1.64 ± 0.53^{c}
DAL	3.40 ± 0.05	3.39 ± 0.05	3.42 ± 0.04	3.38 ± 0.05	3.37 ± 0.04	3.35 ± 0.05	3.39 ± 0.04	3.34 ± 0.03	3.34 ± 0.04
FAWL	0.55 ± 0.05^{a}	0.18 ± 0.08^{c}	0.38 ± 0.11^{b}	0.32 ± 0.09^{b}	0.18 ± 0.09^{c}	0.43 ± 0.08^{a}	0.56 ± 0.07^{a}	0.54 ± 0.07^{a}	0.33 ± 0.08^{b}
ASWL	0.55 ± 0.05^{a}	0.18 ± 0.08^{c}	0.38 ± 0.11^{b}	0.32 ± 0.09^{b}	0.18 ± 0.09^{c}	0.43 ± 0.08^{a}	0.56 ± 0.07^{a}	0.54 ± 0.07^{a}	0.33 ± 0.08^{b}
RAWL	3.06 ± 0.29^{a}	1.09 ± 0.46^{c}	2.15 ± 0.64^{b}	1.74 ± 0.53^{c}	0.34 ± 0.51^{d}	2.40 ± 0.46^{b}	3.08 ± 0.67^{a}	3.05 ± 0.39^{a}	2.00 ± 0.46^{b}
DAWL	17.95 ± 0.19	17.71 ± 0.15	17.54 ± 0.18	18.15 ± 0.15	18.13 ± 0.21	18.11 ± 0.17	18.14 ± 0.14	18.05 ± 0.18	17.36 ± 0.20
FALL	0.03 ± 0.08^{b}	0.05 ± 0.04^{b}	0.03 ± 0.01^{b}	0.09 ± 0.05^{a}	0.02 ± 0.03^{b}	0.08 ± 0.03^{a}	0.04 ± 0.08^{b}	0.04 ± 0.01^{b}	0.04 ± 0.00^{b}
ASLL	0.03 ± 0.08^{b}	0.05 ± 0.04^{b}	0.03 ± 0.01^{b}	0.09 ± 0.05^{a}	0.02 ± 0.03^{b}	0.08 ± 0.03^{a}	0.04 ± 0.08^{b}	0.04 ± 0.01^{b}	0.04 ± 0.00^{b}
RALL	0.02 ± 0.03	0.02 ± 0.02	0.01 ± 0.00	0.03 ± 0.02	0.01 ± 0.01	0.03 ± 0.01	0.01 ± 0.03	0.01 ± 0.00	0.02 ± 0.00
DALL	275 ± 5.65^{b}	271.75 ± 5.74^{b}	270.54 ± 6.07^{b}	263.62 ± 4.83^{c}	284.92 ± 2.57^{a}	281.15 ± 5.60^{a}	286.09 ± 1.62^{a}	286.25 ± 2.42^{a}	277.56 ± 5.16^{b}

^{a,b,c,d} means of different superscripts along the same row are significantly different ($p < 0.05$); Fluctuating Asymmetry of Face Length (FAFL), Relative Asymmetry of Face Length (RAFL), Fluctuating Asymmetry of Wing Length (FAWL), Relative Asymmetry of Wing Length (RAWL), Directional Asymmetry of Wing Length (DAWL), Fluctuating Asymmetry of Leg Length (FALL), Relative Asymmetry of Leg Length(RALL), Directional Asymmetry Leg Length (DALL)

suggested measure or index of developmental instability, low FA is an indicator of small deviation from perfect symmetry (Moller and Manning, 2003). Highest RA was obtained in the Ross strain while highest DA was recorded in the Marshal strain. Also, there was significant effect of strains ($p < 0.05$) on DA of leg length of the chicken with Ross recording highest DA for leg length. However, there was no significant effect of a strain on FA, Anti-Fluctuating Asymmetry and RA of leg length of the chicken. Similar result was obtained by Moller *et al.* (1999), who reported that plumby genotype of broiler chicken may be responsible for the longevity in one part of the body than the other.

Table 2 shows the effect of stocking density on bilateral symmetry of broiler chicken. The results show that the effect of stocking density on bilateral symmetry of face length of the broiler chicken was significant ($p > 0.05$). The bilateral symmetry of wing length was significantly ($p < 0.05$) affected by the stocking density of chicken. Highest FA for wing length was obtained in birds raised on 10 birds.m^{-2}. This implies that highest deviation from perfect symmetry was obtained for birds raised on 10 birds.m^{-2}. Contrarily, Mirtagioglu *et al*. (2013) reported low deviation from perfect symmetry for chickens raised at stocking density of 11 birds.m^{-2}. Also highest DA for wing length was recorded in birds raised at stocking density of 10 birds.m^{-2}. However, there was significant effect ($p < 0.05$) of stocking density on DA of leg length in birds raised at stocking density of 12 birds.m^{-2} which recorded the highest DA for leg length. This is an indication of high importance of rearing factor, condition or environment in production cycle as reported by Skubic (2007).

There were significant effects ($p < 0.05$) of age on bilateral symmetry of face length of the chicken (Table 3). The highest value for bilateral symmetry of face length was obtained when the birds were 56 days old. Age also had significant effect on DA at 56 days old of the broiler chicken. Also the bilateral symmetry of wing length was significantly affected by age. The RA and DA of wing length were significantly affected by age with the highest values obtained when the birds were 56 days old. FA of the wing length was not affected by age, although the highest DA for wing length was obtained when the birds were 56 days old. DA for leg length of the broiler chicken was significantly affected by age with highest DA for leg length. However, the FA and RA were not significantly affected. This result showed that there was increment in the parameters measured as the broiler chicken aged. This was in agreement with the report of Palmer and Strobeck (1992).

Table 4 shows the interaction effects of strain and stocking density on bilateral symmetry of broiler chicken. There were significant ($p < 0.05$) interaction effects of strain and stocking density on bilateral symmetry of broiler chicken. Arbor Acres raised on 14 birds.m^{-2} had highest FA for face length. Ross raised on 12 birds.m^{-2} had the highest RA for face length. Highest FA for wing length was obtained in Ross chicken raised on 10 birds.m^{-2}. Highest FA for leg length was obtained in Arbor Acres raised on 10 birds.m^{-2}, while highest DA for leg length was also obtained in Ross chicken raised on 12 birds.m^{-2}. There was increase in the body parts with the decrease of stocking density in relation to the strain of chicken which confirmed significance on the investigated interaction effects in accordance with the results obtained by Skubic *et al*. (2007), which also indicate importance of this rearing factor in the production cycle. Moller and Manning (2003) have also indicated that animals kept under high stocking density had enhanced FA and lower growth rate.

CONCLUSION

Based on the results of the study it can be concluded that the bilateral symmetry of morphological traits, as estimated by DA, FA and RA, was affected by a strain and stocking density. Also, the bilateral symmetry increased with increase in the age of the birds. Marshall Broiler chicken performed best and they can be raised at 10, 12, and 14 birds.m^{-2}, whilst Arbor Acres and Ross chickens can be raised at 10 and 12 bird.m^{-2}. Strain and stocking density may be claimed as important factors affecting developmental stability of broiler chickens.

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IN VITRO RUMEN MICROBIAL FERMENTATION OF DEHULLED AND INDUSTRIALLY SORTED CHICKPEA (*CICER ARIETINUM* L.) USING GAS PRODUCTION TECHNIQUE

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ABSTRACT

Based on the official statistics of the industries and mines organization, about 7500 tons of wastes in chickpea processing plants are produced annually in the East Azarbaijan province of Iran. In order to determine chemical composition, anti-nutritional factors, and metabolizable energy of chickpea (*Cicer arietinum* L.) by-product (including chickpea pre-screening and chickpea hull), classified random sampling from 10 % of plants was performed first. Then amount of dry matter (DM), organic matter (OM), crude protein (CP), crude fibre (CF), ether extract (EE), neutral detergent fibre (NDF), acid detergent fibre (ADF), and antinutrient including total extractable phenolic compounds (TPC) and total tannin (TT) were determined. There were significant differences ($P < 0.01$) between chemical composition and antinutrient amount of chickpea by-product except for DM and OM. An *in vitro* gas production technique was used to determine the rate and extent of gas production and organic matter digestibility. Amounts of gas production were recorded at 2, 4, 8, 12, 24, 36, 48, 72, 96 and 120 h after incubation. Gas production rate constant and value calculated for OMD and ME of chickpea pre-screening were significantly higher than chickpea hull ($P < 0.01$), but the amount of b and lag time of chickpea hull were significantly higher than chickpea pre-screening ($P < 0.01$).

Key words: chickpea pre-cleaning; gas production; metabolizable energy; chickpea hull

INTRODUCTION

Iran is deficient in protein-rich feedstuffs for livestock and relies heavily on soybean meal imports. For this reason, it is trying to develop its own protein crops and the production of grain legumes has been promoted due to their high level of protein. Chickpeas are one of the oldest and most widely consumed legumes in the world, particularly in tropical and subtropical areas. Chickpeas (*Cicer arietinum*) are a yearly leguminous crop belonging to Fabaceae family. Together with other legumes it has long been one of the most important protein sources of the rural population (Sarno and Stringi, 1980); the dry mature seed is traditionally cooked and eaten with cereals. Chickpea seed has high protein content, about 20 % (Aman, 1979; Khan *et al.*, 1979), and is therefore well suited to human and animal nutrition.

Based on seed colour and site of origin, chickpeas are generally classified as either desi (Indian origin) or Kabuli (Mediterranean origin). The desi type has a smaller seed size and a thicker seed coat than the Kabuli type (Gil *et al.*, 1996). The two chickpeas also differ in their nutrient composition, with the Kabuli type having lower fibre, higher starch and higher fat contents than the desi type (Gil *et al*., 1996). Also chickpea seeds (*Cicer arietinum* L.) are usually grown for human consumption, but approximately 20 % of the production is damaged during harvesting and processing, and considered as by-product sold at low prices for livestock feeding (Ulloa *et al.*, 1988).

After screening, dehulling of chickpea resulted in three fractions including seed coat, cotyledon, and embryonic axe. During the processing of chickpea, 13.8, 1.6, and 84.6 % seed coat, embryonic axe, and cotyledon fractions, respectively, are produced. (Sreerama *et al*., 2010). The rapid development and attention is being

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paid to growing chickpeas in west of Asia. However, as production increases, more chickpeas, which are not suitable for human consumption, will be feed grade and made available as animal feed. Proper utilization of feed grade chickpeas as animal feed will increase the economic returns of growing chickpeas in west of Asia.

The sown area of legume in Iran is over 1.1 and 0.7 million ha is under chickpea cultivation (Sabaghpour *et al.*, 2006). Approximately 7500 ton by-product of chickpea including pre-screening seeds and chickpea hull (known as chickpea by-product) are produced annually in North West Iran. Information on nutritive value of legume in general and chickpea by-product in particular are very low in relation to cereal grains. The aim of the present study was to elucidate the types of nutritional and anti-nutritional compounds and gas production parameters of chickpea by-product.

MATERIAL AND METHODS

Sampling and chemical composition

Dehulling is the first step in the chickpea plants. Dehulling of the legumes results in the production of various types of by-products such as seed coat, embryonic axe fraction, and powder. In the chickpea plants, after dehulling, brown-colored and thick seed coat, powder and embryonic axe fractions were collected by sieving. These fractions were sieved through a 2 mm sieve to collect powder and embryonic axe fractions (passed through the sieve) and seed coats (hulls, remaining on the sieve). The embryonic axe and powder fractions were added to the pre-screening fraction. Samples of chickpea by-product including pre-screening seeds and chickpea hull were collected from 10 % of the chickpea plants of this province according to classified sampling during September to December, 2014.

Samples were then ground to pass through a 1 mm sieve (Retsch Muhle mill, Retch EPP 15X20, Germany), and then were used for chemical analysis and gas production technique. Dry matter content of each sample was determined using a forced-air oven at 105 °C for 24 h. Nitrogen content was determined using the Kjeldahl method (Kjeltec 2300 Autoanalyzer, Foss Tecator AB, Hoganas, Sweden) and CP was calculated as N × 6.25. Ash-free neutral detergent fibre (NDF) was determined, using thermo stable alpha amylase (Sigma A-3306), without sodium sulphite in the ND, according to Van Soest *et al*. (1991). Acid detergent fibre [(AOAC, 2000), ID 973.18] was determined and expressed exclusive of residual ash. Samples were also analyzed for ether extract ([AOAC, 2000], ID 920.39), crude fibre [(AOAC, 2000), 962.09], and ash [(AOAC, 2000), ID 942.05] concentrations. Total carbohydrate was calculated by subtracting the amount of crude protein, ether extract, moisture, and ash from the 1000. Total extractable phenolic compounds (TPC) and total tannin (TT) were determined using procedures of Julkunen-Titto (1985) and Makkar *et al.* (1992), respectively.

Gas production

Three fistulated adult Balochi male sheep (49.5 ± 2.5 kg) were used as rumen liquor donor for gas production technique. Animals were fed a diet to meet their maintenance requirement (NRC, 1985). Sheep were fed a total mixed ration consisting of 0.8 kg DM alfalfa hay and 0.5 kg DM concentrate consisting of 50 % barley grain, 20 % sugar beet pulp, 12 % soybean meal, 15 % wheat barn and 3 % vitamin and minerals (165 g $CP.kg^{-1}$ of DM). The ration was fed twice daily at 08:00 and at 15:00 h (Bilik and Lopuszanska-Rusek, 2010).

Equal volumes of ruminal fluid (about 350 ml) from each sheep were collected via a vacuum pump through fistula before the morning feeding and combined. Rumen fluid was strained through 4 layers of cheesecloth into a pre-warmed CO_2-filled flask. All laboratory handling of rumen fluid was carried out under a continuous flow of CO_2. Incubation of the samples was done using a manual pressure transducer technique.

Incubation of the samples was done using calibrated glass syringes following the procedures of Menke and Steingass (1988). Approximately, 200 mg DM of each sample that were ground to pass through a 2-mm screen in a Wiley mill (Arthur H. Thomas Co., Philadelphia, PA) was weighed into a glass vial (n = 4). Vials were pre-warmed at 39 °C before the injection of 30 ml rumen fluid-buffer mixture (pH = 6.8) into each bottle followed by incubation in a water bath at 39 °C. Following inoculation, during gas pressure reading, vials were briefly and gently rolled to facilitate mixing and to maximize contact of the inocula with the samples, which exhibited a slight tendency to adhere to the glass above or below the gas-liquid interface. Gas pressure measurement were made with a digital pressure gauge (model SEDPGB0015PG5 sensor unit, SenSym, Milpitas, Calif.) having a 0.01 lb/in^2 (or psi; 1 psi = 0.06805 atm) sensitivity. Measurements of the pressure and gas production were done at 2, 4, 6, 8, 12, 24, 36, 48, 72, 96 and 120 h after the incubation. Volume was measured by a graduated syringe (20 mL) also with a coupled needle (0.6 mm). Immediately after inoculation, the initial reading was performed with the aim to standardize the pressure and discard the volume of gas in all bottles. From insertion of the needle into the synthetic rubber stopper, the pressure produced inside the vials was verified in the digital reader. After pressure reading, the volume of gases was determined by pulling the plunger until the transducer pressure returned to zero.

Total gas volumes were corrected for a blank incubation which contained only the buffered rumen

fluid without any barley samples and weight of sample according to the equation proposed by Valentin *et al.* (1999):

$$GP\ (ml.200\ mg.DM^{-1}) = \frac{200\ (V_t - V_0 - V_b)}{W}$$

Where; GP is corrected gas volume (ml), V_t is gas volume recorded in the vial containing sample (ml) at time t (h), V_0 is volume in the vial with sample at 0.0 h of incubation, V_b is gas volume in the vial without sample, and W is weight of sample (mg). Corrected cumulative gas production data were fitted to the exponential model of McDonald (1981): $Y= A(1-e^{(-c(t-lag))})$, where A is the asymptotic gas production (ml); c is the fractional rate of gas production (/h); lag is the initial time delay in the onset of gas production (h) and t is the gas reading time (h). The model allowed for the estimation of a lag phase (Lag) before rapid gas production began.

The parameters A, C and lag time were estimated by an iterative least squares procedure using the NLIN procedure of SAS (2002). Data of 24 h gas production were also used to estimate the organic matter digestibility and metabolizable energy of the samples using the equations of Menke and Steingass (1988) as:

$$ME\ (MJ.kg\ DM^{-1}) = 0.157 \times GP + 0.0084 \times CP + 0.022 \times EE - 0.0081\ CA + 1.06$$

$$OMD\ (\%) = 0.9991 \times GP + 0.0595 \times CP + 0.0181 \times CA + 9$$

Where:

CA is ash in g.kg DM^{-1}; EE is ether extract in $g.kg^{-1}$ and GP is the net gas production (ml.200 mg DM^{-1} in 24 h). Data were statistically analyzed using SAS (1999) software.

RESULTS AND DISCUSSION

Chemical composition of chickpea by-product is presented in Table 1. Nutrient composition and anti-nutritional factors of chickpea pre-screening and chickpea hull, except DM and OM showed significant differences ($P < 0.01$). Chickpea pre-screening contained more crude protein, ether extract but less crude fibre, acid detergent fibre, total carbohydrate, total tannin and total extractable phenolic compounds. Chickpea hull contain higher amount of crude fibre than chickpea pre-screening. Similarly, the ADF, which comprises lignin and cellulose, was also higher in chickpea hull (Table 1). The crude fibre and ADF difference is due to the fact that hull have higher crude fibre and ADF. Dehulling of legumes in general, results in variations in the content of nutrients and anti-nutritional factors in different milled fractions, because the nutrients and anti-nutritional factors in legumes are unevenly distributed in the seed (Shahidi *et al*., 2001).

Results obtained in the present study regarding the chemical composition of chickpea pre-screening confirmed the finding of Abdi and Danesh Mesgaran (2009). These values are similar to the values reported for chickpea (Sreerama *et al*., 2010; Costa *et al*., 2006). Total dietary fibre, was also higher in chickpea hull (Table 1). Ramalho and Portugal (1990) reported the CP values for eight genotypes of chickpea seeds grown at different locations which ranged from 18.2 to 24 % of DM.

The crude protein content of chickpea pre-screening was higher than those reported by Salgado

Table 1: Chemical composition of chickpea by-product (g.kg DM^{-1})

Constituent	Chickpea by-product			
	Chickpea pre-screening	Chickpea hull	s.e.d	P
DM	919	923	4.3	NS
OM	940	927	6.2	NS
CP	279	44	5.4	< 0.01
EE	78	87	1.4	< 0.01
CF	72	178	2.4	< 0.01
Total carbohydrates[a]	502	719	6.5	< 0.01
NDF	351	323	6.6	< 0.01
ADF	96	224	10.1	< 0.01
TT	1	6.5	0.55	< 0.01
TPC	3.4	7.5	0.75	< 0.01

DM, dry matter; OM, organic matter; CP, crude protein; EE, ether extract; CF, crude fibre; NDF, neutral detergent fibre; ADF, acid detergent fibre; TT, total tannin; TPC, total extractable phenolic compounds

[a]By difference as 100 - (moisture + protein + ash + fat). Values are mean ± (standard error of difference); Ns: not significant.

et al. (2001) that showed that the CP level of white and black chickpea seeds were 195.1 and 212.7 g.kg DM^{-1} respectively. This could be due to higher embryonic axe that was produced during chickpea dehulling and added to chickpea pre-screening by product. Sreerama *et al.* (2010) reported that the protein content of embryonic axe fractions in chickpea and horse gram were higher than seed coat fractions. Who also reported that seed coat fractions had the lowest recorded protein content of 7.3 % in chickpea and 9.1 % in horse gram.

Crude fibre content was higher in seed coat fractions than pre-screening fraction. These results are similar to the values reported for chickpea (Sreerama *et al*., 2010) and beach pea (Shahidi *et al*., 2001). The presence of high crude fibre in food material is reported to decrease dry matter digestibility in animals (Devendra, 1995).

The levels of phenolic compounds were higher in seed coat fractions than pre-screening fraction. Similar results were observed by Sreerama *et al*. (2010). These results indicate that phenolic compounds are mostly concentrated in the seed coat fractions and might be easily removed by dehulling. Phenolic compounds, which are abundantly present in the seed coats of legumes, are one of the most important groups of secondary metabolites in plants having anti-nutrient properties (Sreerama *et al*., 2010). Tannins may form a less digestible complex with dietary proteins and may bind and inhibit the endogenous protein, such as digestive enzymes (Kumar and Singh, 1984). Tannin can adversely affect the microbial and enzyme activities (Singleton, 1981; Lohan *et al.*, 1983; Barry and Duncan, 1984; Makkar *et al.*, 1989).

The content of phenolic compounds in chickpea pre-screening fractions of chickpea is lower than those reported for beach pea (Shahidi *et al*., 2001), cowpea, pea, pigeon pea, and chickpea cotyledon fraction (Reddy *et al*., 1985). Phenolic compounds usually form insoluble complexes with protein, thereby interfering with their bioavailability (Liener, 1994). However, these phenolic compounds have been reported to act as antioxidants by preventing oxidative stress that causes diseases such as coronary heart disease, some types of cancer, and inflammation (Tapiero *et al*., 2002). Because the content of phenolic compounds is higher in seed coat and embryonic axe fractions of chickpea and horse gram, they are likely to have antioxidant activity.

Cumulative gas production profiles from the *in vitro* fermentation of chickpea pre-screening and chickpea hull are shown in Figure 1. The cumulative volume of gas production increased with increasing time of incubation. Gas produced after 96 h incubation ranged between 342.43 and 403.98 ml per g of dry matter. Cumulative gas production was comparable to those reported by Maheri-Sis *et al.* (2007). At all incubation times cumulative gas productions (ml) of chickpea pre-screening was significantly ($P < 0.05$) higher than chickpea hull.

Gas production parameter and calculated amount of organic matter digestibility (OMO) and metabolizable

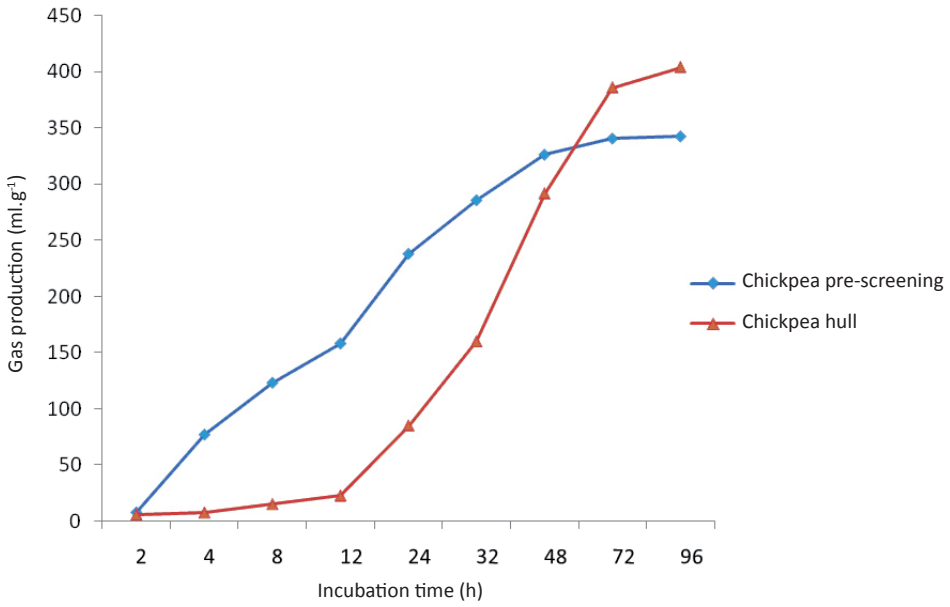

Fig. 1: Gas production of chickpea pre-screening and chickpea hull when incubated with rumen fluid at different incubation times

Table 2: Gas production parameters, organic matter digestibility (OMD)* and metabolizable energy (ME) content of chickpea by-product**

Item	Chickpea	Chickpea hull	P
	pre-screening		
A (ml)	348.0 ± 12.1	467.5 ± 12.5	< 0.01
c (ml.h^{-1})	0.050 ± 0.0022	0.024 ± 0.0023	< 0.01
L (h)	0.7 ± 0.33	5.25 ± 1.3	< 0.01
OMD (%)	59.1 ± 0.42	42.1 ± 1.5	< 0.01
ME (MJ.kg^{-1})	8.95 ± 0.074	6.5 ± 0.2	< 0.01

A, asymptotic gas production (ml); c, fractional rate of gas production (ml.h^{-1}); lag, the initial time delay in the onset of gas production (h)
*OMD = 0.9991 Gas + 0.0595 CP + 0.0181 CA + 9
**ME = 0.157 Gas + 0.0084 CP + 0.022 EE – 0.0081 CA + 1.06

energy (ME) are presented in Table 2. The amount of c and value calculated for OMD and ME of chickpea pre-screening were significantly higher than chickpea hull ($P < 0.01$). This might be due to difference in chemical composition and volume of gas production in the first 24 h. Lag time of chickpea hull was significantly higher than chickpea pre-screening ($P < 0.01$).

CONCLUSION

It was concluded that the by-products evaluated in the present experiment had a potential to use as suitable feed in ruminant rations. There is no apparent anti-nutritive factor other than tannins, whose effect must be further tested. However, future feeding trials will be proposed to evaluate the effect of this by-product in ruminant production.

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