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EFFECT OF TAURINE ON BOVINE SPERMATOZOA MOTILITY PARAMETERS FOLLOWING CRYOPRESERVATION

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ABSTRACT

The use of artificial insemination in animal production and especially in cattle breeding is an important intensification factor. For the purpose of maximal use of genetic potential, importance is always put on the creating conditions for effective insemination. The aim of this work was to analyze the effect of the taurine addition to bovine ejaculates in the process of cryopreservation on the spermatozoa motility.
Taurine was dissolved in a saline to obtain various concentrations (50, 100, 200, 400 and 600 mM) and added to the extender. Fresh ejaculates collected from six breeding bulls were used in the experiment. Ejaculates were placed to the prepared extender. The control sample consisted of the extender only. After four hours of exposure at 4 °C, the ejaculates were put into straws with a volume of 0.5 ml. The semen was then exposed to freezing temperature of nitrogen vapour for 10 min. The straws were stored for one month in liquid nitrogen at -196 °C. Thereafter, the spermatozoa motility parameters were analyzed using the CASA method. This assay was repeated four times in the intervals of 0, 30, 60 and 90 min at 39 °C. The correlation between the time after thawing and spermatozoa motility shows a regressive character in the curve. In semen doses with taurine at 200 mM significant increase in the motility and progressive motility, in comparison to the control sample, was recorded. The results show that the addition of taurine increases total motility and progressive motility of bovine spermatozoa.

Key words: spermatozoa; cryopreservation; taurine; CASA; bull; artificial insemination; motility

INTRODUCTION

The spermatozoa is a male gamete almost devoid of cytoplasm with large nucleus, containing haploid amount of highly condensed chromosomes, an acrosome, responsible for interaction and penetration of oocyte and series of mitochondria located at the anterior region of the flagellum (Eddy and O´Brien, 1994). Mitochondria produce ATP mainly for the purpose of maintaining motility of the spermatozoa, while Golgi apparatus and endoplasmic reticulum work on maintaining integrity of the cell membrane (Medeiros *et al*., 2002). Not more than 7 % of spermatozoa are present in the ejaculate. Spermatozoa are protected and nourished by seminal plasma. Bull seminal plasma originates from the urethral glands, the ampullary glands and the seminal vesicles (Rothschild and Barnes, 1954). According to Massányi *et al.* (2003), there are significant differences in composition of seminal plasma among different animals due to differences in structure and function of reproductive system.

Artificial insemination (AI) is the mostly used biotechnology method in cattle reproduction (Foote *et al.*, 2002). Use of the AI has a great potential in breeding of domestic animals. Its benefits come out of potential use of genetic material from small number of superior sires (Watson, 2000; Maxwell and Watson, 1996). AI allows crossbreeding, which results in hardening traits in milk and meat production (Unal *et al*., 2006).

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Cryopreservation is a method of storing cells and tissues in liquid nitrogen, which have use in different fields of biology, medicine and agriculture (Andrabi and Maxwell, 2007). The use in agriculture is focused on genetic improvement of domestic species and preserving rare plant species and rare breeds of animals (Holt, 1997). Arav *et al*. (2002) state that vitrification is the most beneficial method of cryopreservation of spermatozoa because of decreasing cold shock by rapid freezing. Cryopreservation is a method which slows the cellular metabolic activity but restarts it after thawing (Medeiros *et al*., 2002; Mazur, 1984). Intracellular ice crystals are formed during cryopreservation, what results in different damages to spermatozoa, such as a cytoplasm fracture, abnormalities in the cytoskeleton and in genome-related structures (Isachenko, 2003). According to Del Maestro (1980), decline in motility, functional integrity of spermatozoa membranes and fertility can be attributed to the action of reactive oxygen species (ROS), namely hydrogen peroxide (H_2O_2) and superoxide anion radical (O_2-). Protection against ROS and improved spermatozoa motility after addition of an antioxidant like taurine to the extender was proved by Bucak and Tekin (2007).

Extenders for semen cryopreservation must have suitable osmolality, adequate pH and buffering capacity to protect spermatozoa from cryogenic injury (Salamon and Maxwell, 2000). Cryoprotectants used as extenders are either penetrating or non-penetrating the cell membrane. Taurine is, therefore, classified as a non-penetrating and acts extracellularly (Barbas and Mascarenhas, 2009; Purdy, 2006).

Taurine is an organic acid, which contains sulphur. Molecular structure of taurine is very similar to γ-aminobutyric acid (GABA), which is the main neurotransmitter in brain (Huxtable, 1992). Taurine has cytoprotective abilities which emerge from the ability to detoxicate, osmoregulate and maintain calcium homeostasis (Devi *et al*., 2008). Sinha *et al*. (2008) mention that mechanisms of taurine cytoprotective abilities are still not well-investigated, however taurine can be perceived as an antioxidant due to its efficiency in efflux of free radicals along with maintaining the cell membrane permeability exposed to ROS.

The aim of this study was to determine spermatozoa motility in semen doses with various concentrations of taurine in comparison to conventionally produced semen doses without taurine.

MATERIAL AND METHODS

Animals and semen collection

Semen samples were obtained from 6 breeding bulls. All bulls were held and maintained under usual housing and feeding conditions. Ejaculates were collected to artificial vagina maintained at temperature of 38 – 40 °C. Consistency of ejaculates was determined spectrophotometrically.

Semen processing

The amount of extender added to ejaculates was calculated according to its consistency to reach at least $20x10^6$ spermatozoa per each insemination dose. Extender, consisted of egg yolk, glycerine, fructose, citric acid, Tris, *aqua pro injectione*, antibiotics Norostrep® a Linco-Spectin® and taurine (Taurine ≥ 99 %, Sigma Aldrich, Bratislava, Slovakia) dissolved in physiological solution, was added to experimental samples. Concentrations of taurine in physiological solution were 50 mM, 100 mM, 200 mM, 400 mM and 600 mM. Control sample (without taurine) was labeled with letter X and experimental samples were marked according to increasing taurine concentration with letters A, B, C, D, E. Diluted ejaculates were cooled down to 4 °C for four hours. Ejaculates were filled into straws with the volume of 0.5 ml. Consecutive cooling of straws at 4 °C lasted for 10 min. Subsequent freezing involved 10 min exposure to liquid nitrogen vapor, afterwards the straws with sperm samples were stored in liquid nitrogen for a one month.

Semen evaluation

Semen analyses were performed using the CASA method with SpermVision software (Minitub, Tiefenbach, Germany) and the microscope Olympus BX 51 (Olympus, Japan). Five straws of control group and five straws of each experimental concentration were analyzed to achieve the most authentic results. After thawing samples were placed to thermostat at the temperature of 39 °C and afterward it were transferred to Makler counting chamber (10μm, Sefi–Medical Instruments, Germany). Measurements of spermatozoa motility were repeated three-times every half an hour (Time 0, 30, 60, 90) and the tested samples were stored in a thermostat at 39 °C. The following spermatozoa characteristics were assessed: motility (MOT), progressive motility (PRO), beat cross frequency (BCF), curvilinear velocity (VCL) and amplitude of lateral head displacement (ALH).

Statistical analysis

For the comparison of the CASA results in certain time intervals with the focus on effects of extenders, ANOVA and Dunnett´s comparative test were applied using GraphPad Prism 5 (GraphPad Software Inc., USA). Control sample was considered to be 100 % and, at the same time, a basic comparison value for all experimental samples was set. All statistical tests were carried out at levels of significance at $p < 0.05$, $p < 0.01$ and $p < 0.001$.

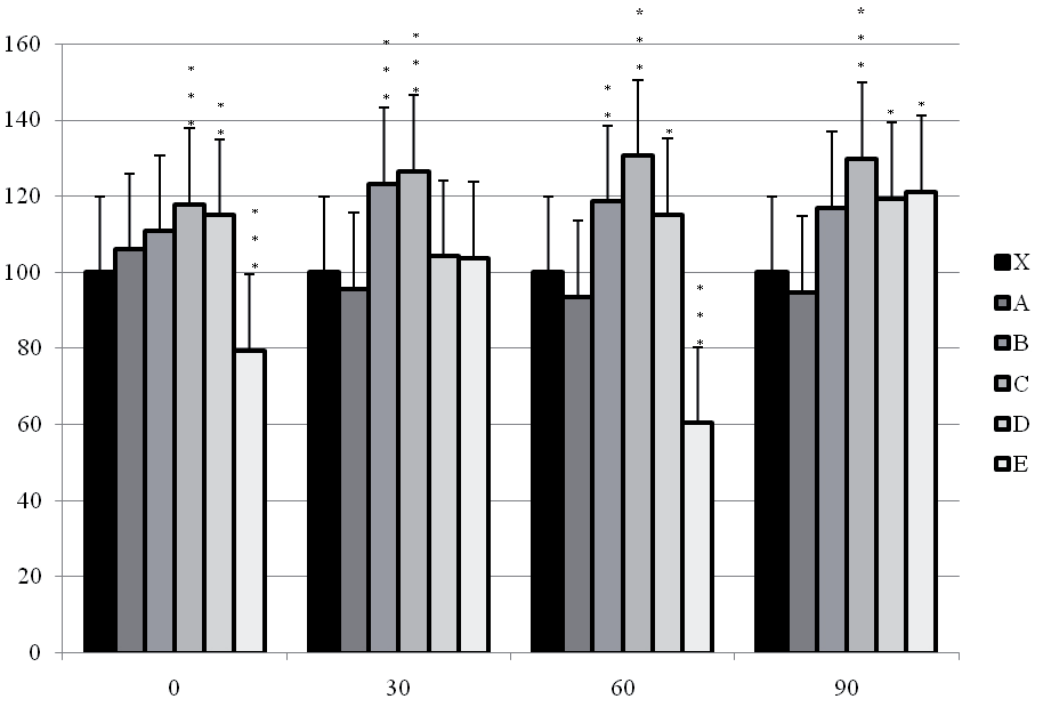

Fig. 1: Spermatozoa motility [control = 100 %] in different taurine concentrations at various time intervals (min.) (*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001)

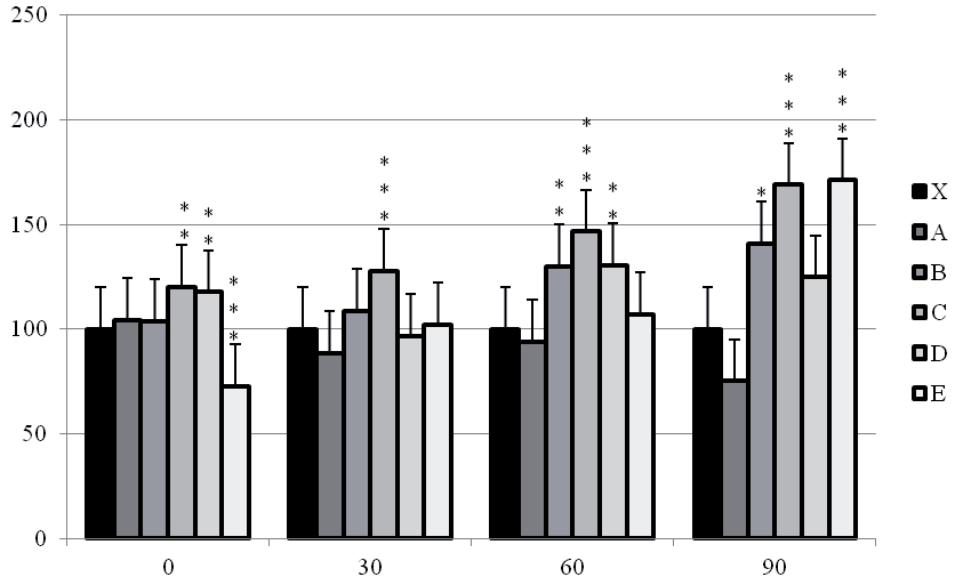

Fig. 2: Spermatozoa progressive motility [control = 100 %] in different taurine concentrations at various time intervals (min.) (*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001)

RESULTS

Post-thawing differences between conventionally protected spermatozoa and 5 various taurine concentrations of experimentally protected spermatozoa were assessed using CASA method.

Spermatozoa motility (Figure 1), measured immediately after thawing, was in control sample lower than in experimental samples, except for the E group (the highest taurine concentration). Significance difference was found for the C ($p < 0.001$) and D ($p < 0.01$) concentration. After 30 min incubation

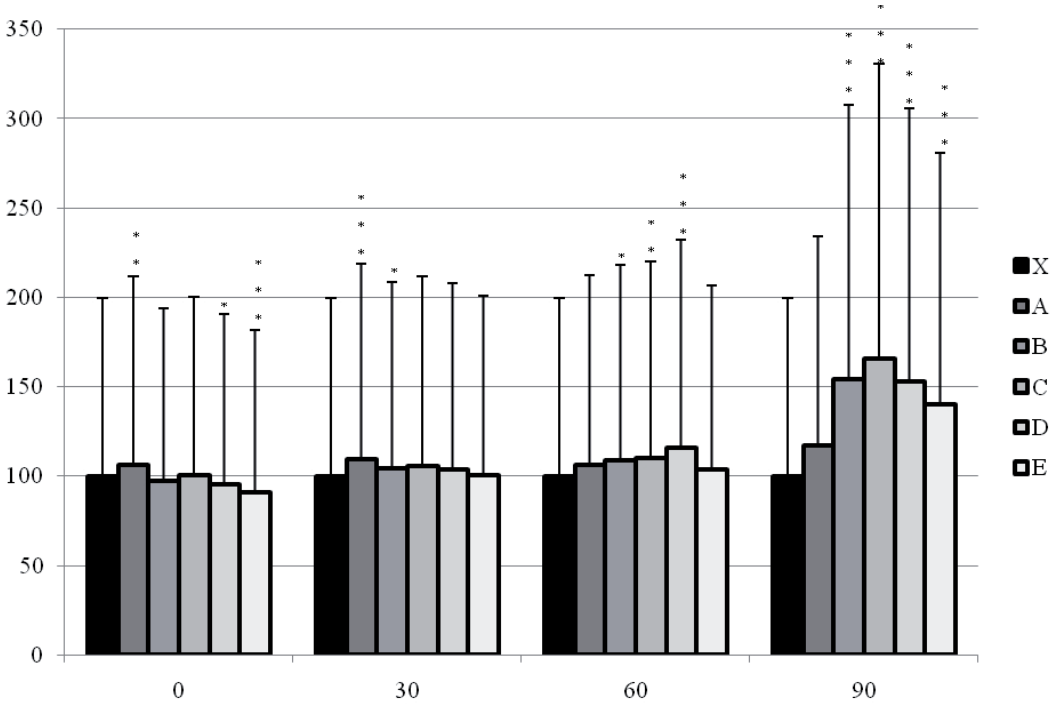

Fig. 3: Beat cross frequency [control = 100 %] in different taurine concentrations at various time intervals (min.) (*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001)

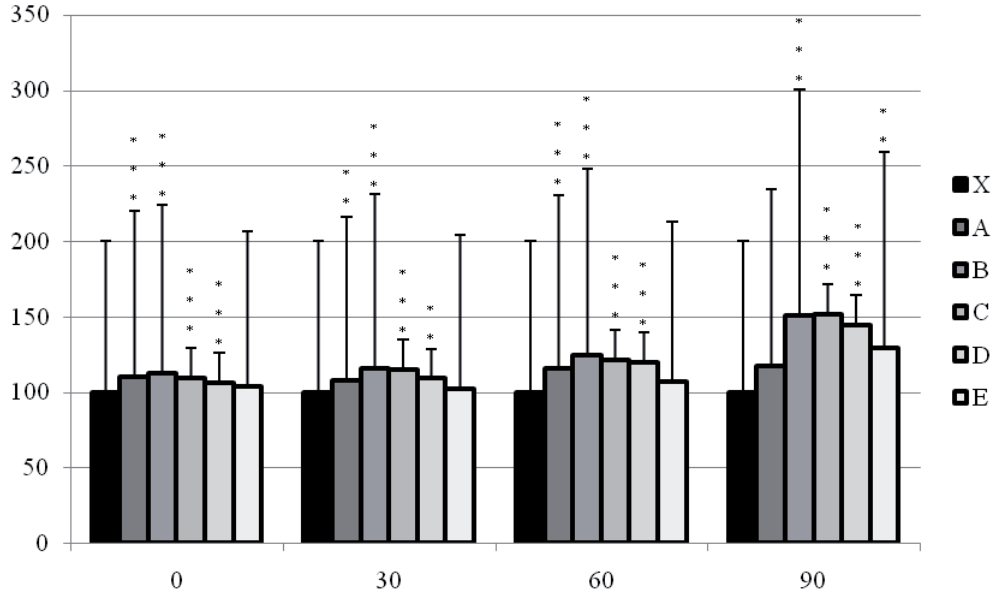

Fig. 4: Curvilinear line velocity [control = 100 %] in different taurine concentrations at various time intervals (min.) (*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001)

significantly higher motility ($p < 0.01$) was detected in samples B and C. Significant increase in the motility was found for concentrations B ($p < 0.01$), C ($p < 0.001$) and D ($p < 0.05$) 60 min after thawing. After 90 min of incubation a higher motility was recorded for C ($p < 0.001$), D and E ($p < 005$) groups.

Spermatozoa progressive motility (Figure 2) at the beginning of the assessment reflected results of spermatozoa motility. Only E concentration showed lower progressive motility compared to control ($p < 0.001$). Higher progressive motility ($p < 0.01$) was recorded for C and D groups. After 30 min incubation a significant increase was recorded only for C concentration ($p < 0.001$). After 60 min significantly higher spermatozoa progressive motility was found at B ($p < 0.05$), C and E concentrations ($p < 0.001$). The further 30 min incubation at 39 °C resulted in significantly higher progressive motility in groups B ($p < 0.05$), C and E ($p < 0.001$).

Beat cross frequency (Figure 3) at initial time showed positive effect of taurine on spermatozoa in sample B ($p < 0.01$). Negative effect was proven in samples D ($p < 0.05$) and E ($p < 0.001$). Following 30 min incubation higher BCF values in comparison to control were recorded for A ($p < 0.001$) and B ($p < 0.05$) concentrations. The further 30 min of incubation resulted in higher BCF at concentrations B ($p < 0.05$), C ($p < 0.01$) and D ($p < 0.001$). After 90 min incubation significantly higher values were recorded for samples C ($p < 0.001$), D ($p < 0.05$) and E ($p < 0.05$).

Velocity of spermatozoa in curvilinear line (VCL; Figure 4) was higher in all experimental samples compared to the control sample for all time periods. Significant differences with control group ($p < 0.001$) during the first assessment of VCL was found in samples A, B, C, D. Incubation in a thermostat for 30 min positively affected all experimental samples. Incubation for 90 min resulted in significantly higher VCL in samples B (+41 %; $p < 0.001$), C (+69 %; $p < 0.001$), D (+25 %; $p < 0.001$) and E (+71 %; $p < 0.01$).

Amplitude of lateral head displacement (ALH; Figure 5) copied previous spermatozoa motility parameters with significant differences (sample B – $p < 0.05$; C – $p < 0.001$). After 30 min incubation significant increase was detected for the group B ($p < 0.05$) and C ($p < 0.05$). No significant differences were found after 60 min of incubation. After 90 min incubation positive effect of taurine was recorded for samples B (+24 %; $p < 0.01$), C (+26 %; $p < 0.001$) and D (+27 %; $p < 0.001$).

DISCUSSION

Differences between fresh and frozen-thawed semen are significant, what is reflected in reduced fertility (Salamon and Maxwell, 1995). It is also proved that frozen-thawed semen contains only 50 % of motile spermatozoa in comparison with fresh semen (Salamon and Maxwell, 2000).

Massányi *et al*. (2011) analyzed effect of various additives on bull spermatozoa motility and, on the basis of CASA results, determined that substances with

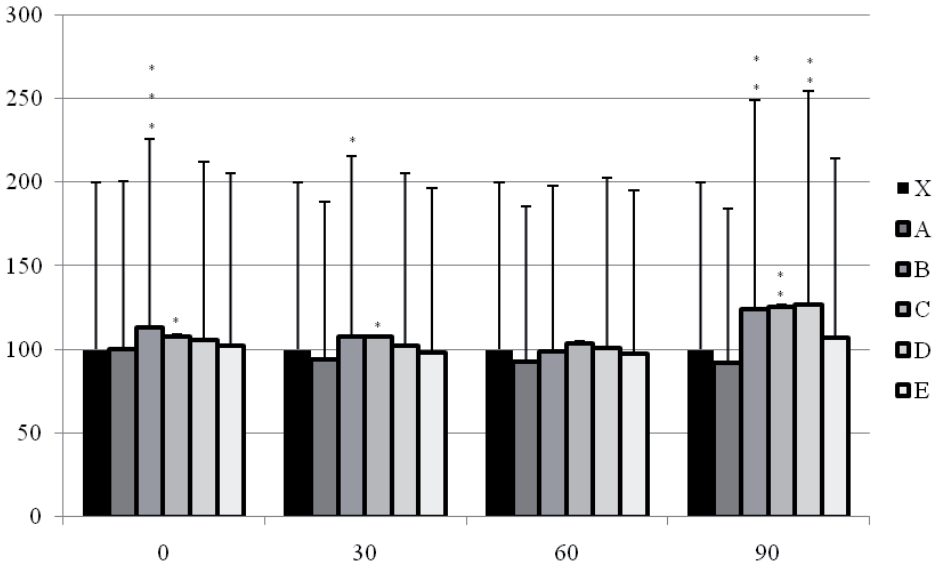

Fig. 5: Amplitude of lateral spermatozoa head displacement [control = 100 %] in different taurine concentrations at various time intervals (min.) (*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001)

antioxidant action have positive impact on spermatozoa motility, what is in accordance with our data. Bucak and Tekin (2007) studied effect of taurine on ram semen during cryopreservation. Experimental sperm samples, enriched with 50 or 100 mM taurine showed, that taurine contributes to creation of an ideal environment for the spermatozoa, in comparison with conventional TRIS-based egg yolk extender. Motility parameters measured at time intervals of 0, 6, 24 and 30 hours are in concert with our data on bovine spermatozoa recorded following 0, 30, 60, 90 min after thawing. Although the time intervals are different, the trend of higher motility in taurine protected spermatozoa was proved. Significant differences in taurine-treated cryopreserved semen were demonstrated also by Chhillar *et al*. (2012). In addition to motility analysis membrane integrity and intracellular calcium tests were performed. The results show that taurine antioxidant action may be the reason of higher motility in experimental sperm samples.

Taurine has multiple biological and metabolic functions as an antioxidant that conjugates biliary acids, detoxifies some xenobiotics and modulates intracellular calcium levels. Taurine preserves the motility of the spermatozoa, supports their capacitation, improves the chances of success of fertilization and the early embryonic development. This is why it can be found in some culture media for *in vitro* fertilization (Bidri and Choay, 2003; Guérin and Ménézo, 1995).

Intracellular taurine is maintained at high concentrations in a variety of cell types and alteration of cell taurine levels is difficult. The role of taurine within the cell appears to be determined by the cell type. Recent and past studies suggested that taurine might be a pertinent candidate for use as a nutritional supplement to protect against oxidative stress, neurodegenerative diseases or atherosclerosis (Bouckenooghe *et al*., 2006). Fan *et al*. (2009) and Yang *et al.* (2010) reported that male accessory sex glands are able to synthesize taurine through the cysteine sulfinate decarboxylase (CSD) pathway. Also Li *et al*. (2006) reported that male genital organs have the function to produce taurine through the CSD pathway, although quantifying the relation of CSD expression to taurine synthesis and the exact functions of taurine in male genital organs still need to be elucidated in future studies.

Taurine and hypotaurine have been found in spermatozoa and seminal plasma of numerous species and are known to have beneficial effects on spermatozoa characteristics in mammals. Previous study investigated the effect of taurine on rabbit spermatozoa motility in vitro (Kročková *et al*., 2013). Total spermatozoa motility and progressive motility were evaluated immediately after samples preparation, after 2 hours of incubation and after 24 hours of incubation. The results confirm that the addition of taurine increases motility and progressive motility of rabbit spermatozoa. With the increase of its concentration and the length of incubation the parameters of motility were stimulated almost in all experimental groups (Kročková *et al*., 2013). Also, significant amounts of taurine and hypotaurine were found in spermatozoa, seminal plasma and epididymal flushing fluid (Buff *et al*., 2001).

Holmes *et al*. (1992) determined taurine and hypotaurine levels in human spermatozoa and seminal fluid. Sperm hypotaurine content was significantly correlated with the spermatozoa morphology, relative forward progression, the percentage of motile spermatozoa and the total number of spermatozoa in the ejaculate. Oppositely, sperm taurine content was negatively correlated with these parameters. Hypotaurine, as an antioxidant, may play an important role in protecting spermatozoa from reactive oxygen species. Higher concentrations of taurine in the spermatozoa of infertile men suggest that accelerated oxidation of hypotaurine to taurine may accompany the observed decline in other spermatozoa parameters. The results of our experiments show, that the addition of taurine increases total motility and progressive motility of bovine spermatozoa. The elevation of taurine concentrations and the length of incubation resulted in the stimulation of the sperm motility almost in all experimental groups.

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HIGH DIETARY LEVELS OF ZINC FOR YOUNG RABBITS

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ABSTRACT

The effects of orally administered zinc from inorganic or organic sources on selected parameters of meat quality were the priority of this study. A total of 96 weaned rabbits (35th day of age, both male and female) were divided into 4 groups (control C and 3 experimental groups – 1EG, 2EG and 3EG) with 24 animals in each group. Maternal albinotic line (crossbreed New Zealand White, Buskat Rabbit, French Silver) and paternal acromalictic line (crossbreed Nitra‘ Rabbit, Californian Rabbit, Big Light Silver) were used. The feed mixture was additionally administered as follows: in 1st experimental group 1EG by a dose of 27.47 g $ZnSO_4.H_2O$ (zinc sulphate monohydrate), in 2nd group (2EG) by a dose of 38.46 g Glycinoplex-Zn and in 3rd group (3EG) dose of 66.67 g Bioplex-Zn, per 100 kg each. They were fed with complete granulated mixtures *ad libitum* and had free access to water via a nipple drinker. Dietary supplementation of rabbit with zinc was carried out to determine its effects on growth of live weight and consumption of feed per unit of live weight growth. On 91st day of age (6 weeks after all experimental procedures), 6 animals from each group were slaughtered and sampled for testing in the morning hours. Meat quality was analyzed from each sample of *Musculus longissimus dorsi* (MLD) (50 g) for parameters characterizing the content of nutrients (content of water, proteins, fat, amino acids and fatty acids composition) and processing technology parameters (electric conductivity, pH, colour). The amino acids and fatty acids contents noted in this study indicate statistically insignificant changes ($p \leq 0.05$). This study suggests that lean rabbit meat could be a high quality protein source due to its well-balanced essential amino acid composition. The growth rate in all groups was independent on zinc treatment. A weak influence of Glycinoplex-Zn on animal health was also noted. Zinc supplementation raises levels of cholesterol, water holding capacity and energy value, and lowers the value of pH in *longissimus dorsi* muscle compared to rabbits fed the control diet. Supplementation with Glycinoplex-Zn (100 mg of zinc) evoked a 25 % mortality rate respectively in comparison to 8.3 % of the Bioplex-Zn supplemented with frequent *Pasteurella* infections. These effects were not observed in rabbits fed with other diets.

Key words: rabbits; zinc; meat quality

INTRODUCTION

Zinc (Zn) is both an essential nutrient and a possible pollutant in animal production system. While it is generally supplemented at low levels in animal diets (less than 200 $mg.kg^{-1}$ in complete feeds), it is under scrutiny due to potential accumulation in the environment. This explains why international regulations strictly limit maximum supplementation levels in animal feeds. The role of micro-minerals in health cannot be over emphasized; zinc has been a modifier of wide spectrum of biological activities. Its deficiency has been related to various dysfunctions and alterations of normal cell metabolism. In this study, supplementation of rabbits with zinc salt was conducted to determine its effects on reproductive performance and growth rate following improvement in the quality and quantity of non-traditional meat as a source of protein for the

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consumers (Underwood, 1977; Alikwe *et al.* 2011). The role of zinc in the animal organism has begun to gain special attention. Zinc participates actively in protein synthesis and carbohydrate metabolism. The discovery that the enzyme carbonic anhydrase contains 0.33 % of zinc in its molecule is considered the first acceptable explanation of Zn mechanism of action. After that, many other enzymes have been identified to contain zinc: alcohol dehydrogenase, carboxipeptidase and DNA-polymerase, the latest being fundamental in cell division process. This mineral stabilizes the quaternary structure of enzymes; large quantities of zinc were found to provide stability to the structures of RNA, DNA and ribosomes (Prask and Plocke, 1971, quoted by Mc Dowel, 1992). Zinc requirement for rabbits is 30 – 60 mg.kg^{-1} dry matter, with suggestion of higher levels for breeders (Mateos and Blas, 1998). It is also essential in cell division, synthesis and stability of DNA (Evenson *et al*., 1993), as well as in cellular differentiation. Animals need microelements in small quantities, and these microelements play an important role in virtually all physiological and biochemical processes, from bone structure to maintaining the structure of proteins and lipids. Microelements are provided to animals in food, by special supplementation (premixes) or in water. In the intensive production, their addition is obligatory, since it has been the only way to provide them in sufficient quantities required for optimum health and production results (Pajtáš *et al*., 2009; Chrastinová *et al*., 2014; Gralak and Chrenková, 2014).

Minerals activate enzymes and they are essential cofactors of metabolic reactions, and function as carriers of proteins, regulate digestion, respiration, water balance, muscle response, the neural transmissions, influence and maintain skeletal strength, balance pH, and even mental balance, protect against disease, act as antagonists or synergists of other elements and play a vital role in the resistance, adaptation and evolution of new races and lines (Anke and Szentmihalyi, 1985; Haenlein, 1987).

Regardless of the fact that certain microelements are present in sufficient quantities in food, subclinical or clinical symptoms of their deficit appear, because their availability varies, or the microelement is present in a form that cannot be used. It was established that the presence of certain substances in food (phytic acid and oxalic acid), as well as interaction with other nutrients in the digestive tract influences resorption mechanisms. Resorption of microelements is not dependent only on their content in food, but also on the animal‘s age, on electrochemical reactions in the intestine, and on the form of the microelement. Salts of minerals are most frequently used, namely oxides, carbonates, chlorides, and sulphates. Today, in addition to inorganic forms of minerals, the use of so-called „chelate“ forms, i.e. organically bonded microelements is becoming more frequent. The aim of this study was to reveal the effects of orally administered zinc from inorganic or organic sources on selected parameters of meat quality.

Table 1: Composition and nutrient content of granulated diet for growing rabbits

Ingredients	%	Chemical analysis	Original matter (g.kg^{-1})
Lucerne meal	36	Crude protein (N*6,25)	177.99
Extracted sunflower meal	5.5	Crude fibre	146.97
Extracted rapeseed meal	5.5	Fat	36.08
Wheat bran	9	Ash	97.32
Oats	13	Starch	129.05
Malt sprouts	15	Organic matter	847.49
DDGS	5	Acid detergent fibre (ADF)	185.13
Sodium chloride	0.3	Neutral detergent fibre (NDF)	315.49
Mineral and vitamin mixture*	1.7	Calcium	9.73
Barley grains	8	Phosphorus	6.94
Limestone	1	ME (MJ.kg^{-1})	11.35

*Premix contains per kg: calcium, 6.73 g; phosphorous, 4.13 g; magnesium, 1.90 g; sodium, 1.36 g; potassium, 11.21 g; iron, 0.36 g; copper, 0.03 g; selenium, 0.2 mg. Vitamin mixture provided per kg of diet: Vitamin A 1500000 IU; Vitamin D3 125000 IU; Vitamin E, 5000 mg; Vitamin B1, 100 mg; Vitamin B2, 500 mg; Vitamin B6, 200 mg; Vitamin B12, 0.01 mg; Vitamin K3, 0.5 mg; biotin,10 mg; folic acid, 25 mg; nicotinic acid, 4000 mg, choline chloride, 100000 mg; DDGS: dried distillers grains with solubles

MATERIAL AND METHODS

A total of 96 weaned rabbits (35th day of age, both male and female) were divided into 4 groups (control C and 3 experimental groups – 1EG, 2EG and 3EG) with 24 animals (in a replicated 6 x 4) in each group. The rabbits of meat line M91, maternal albinotic line (crossbreed New Zealand white, Buskat rabbit, French silver) and paternal acromalictic line (crossbreed Nitra's rabbit, Californian rabbit, Big light silver) were used in this experiment. The experiment lasted 48 days. Rabbits were kept in the standard cages, 2 animals per cage. A cycle of 16 h of light and 8 h of dark was used throughout the experiment. Temperature and humidity in the building were recorded continuously by a digital thermograph positioned at the same level as the cages. Heating and forced ventilation systems allowed the building air temperature to be maintained within 22 ± 4 ˚C throughout the experiment. Relative humidity was about 70 ± 5 %. The rabbits were fed with a commercial diet (pellets of 3.5 mm in diameter). The ingredients and chemical composition of this diet is presented in Table 1.

The feed mixtures for other testing groups (1EG, 2EG and 3EG) were additionally administered before homogeneity of feed mixture: in 1st experimental group by a dose of 27.47 g $ZnSO_4.H_2O$, in 2nd group (2EG) by a dose of 38.46 g Glycinoplex-Zn and in 3rd group (3EG) by a dose of 66.67 g Bioplex-Zn per 100 kg. They were fed with complete pelleted diets *ad libitum* (Table 2). Animals had free access to water via a nipple drinker. In this study, institutional and national guidelines for the care and use of animals were followed, and all experimental procedures involving animals were approved by the ethical committee. The ME content was calculated by the equation of Wiseman *et al*. (1992). Chemical analyses were conducted according to AOAC (1995) with the considerations given by Gidenne *et al*. (2001) for dry matter (DM), crude protein (CP), crude fibre (CF), crude fat, nitrogen free extract, ash and organic matter. Neutral detergent fibre (NDF) and acid detergent fibre (ADF) were analysed sequentially (Van Soest *et al*., 1991) with a thermo stable amylase pre-treatment and starch according to the alpha-amyloglucosidase method.

Body weight and feed consumption were registered weekly. On 91st day of age (6 weeks after all experimental procedures), 6 animals from each group were slaughtered and sampled for testing in the morning hours. After electro-stunning (90 V for 5 s), rabbits were slaughtered in an experimental slaughterhouse by cutting the carotid and jugular veins, bleeding out; the MLD samples were separated by removing the skin and connective tissue chilled and stored for 24 h at 4 °C until physico-chemical analysis. The ultimate pH was determined after 24 h (*post mortem*) using a Radelkis OP-109 (Jenway, England) with a combined electrode penetrating 3 mm into samples. The electrical conductivity ($\mu S.cm^{-1}$), defined as locations of muscles, was evaluated using PMV 51 (Tecpro GmbH, Germany), colour characteristics were expressed by CIE L*a*b system

Table 2: Performance of rabbits in response to dietary supplementation with zinc from inorganic or organic sources ($\bar{x}$ ± SD)

Parameters (n = 24)	C	1EG	2EG	3EG	C	1EG	2EG	3EG
	Adaptation period				Experimental period			
Feed intake g	91.73	89.35	89.74	92.74	133.93	132.66	130	132.83
Initial weight g	1316 ±103	1362 ±59	1374 ±156	1335 ±14	1637 ±119	1633 ±33	1663 ±183	1638 ±93
Final weight g	1637 ±119	1633 ±33	1663 ±183	1638 ±93	2971 ±160	3004 ±229	3049 ±207	2954 ±189
Daily weight gain $g.day^{-1}$	41.61	38.37	41.37	43.36	31.76	32.63	33.00	31.34
Feed conversion ratio $g.g^{-1}$	2.20	2.31	2.16	2.14	4.23	4.08	4.20	4.26
Carcass yield %	-	-	-	-	59.24 ±0.78	59.41 ±1.59	60.12 ±0.45	58.37 ±3.38
Mortality (n)	0	0	1	2	0	0	5	0

Control-C; 1EG – with $ZnSO_4.H_2O$; 2EG – with Glycinoplex-Zn; 3EG – with Bioplex-Zn

(lightness-L*, 0: black and 100: white), (redness and greenness-a*; yellowness and blueness-b*) using a Lab. Miniscan. Lightness measurements at room temperature were also done. The content of water, protein and fat were estimated using an INFRATEC 1265 (Germany) spectroscope and expressed in $g.100.g^{-1}$; and from these values, the energy value was calculated:

EV ($kJ.100.g^{-1}$) = 16.75 x protein content + 37.65 x fat content.

The water holding capacity was determined by the compress method at constant pressure (Hašek and Palanská, 1976). The fatty acid (FA) composition of MLD samples were determined (Ouhayoun, 1992) by gas chromatography of fatty acid methyl ester (FAME) on GC 6890 N (Agilent Technologies, Switzerland). Results were expressed as percentages of total fatty acids. Fatty acid composition varies a lot and is expressed as share of SFA (saturated fatty acid), MUFA (monounsaturated fatty acid), PUFA (polyunsaturated fatty acids), P/S and n6/n3 index. The amino acids composition of diet was analyzed by ion-exchange chromatography on AAA (Ingos Prague, Czech Republic) after acid hydrolysis with 6 M HCl, methionine and cystine after oxidation hydrolysis. Weight of feed mixture was checked daily and average daily weight gain and feed conversion were calculated mathematically as well as mortality at the end of the experiment.

The results were expressed as mean ± standard deviation (SD); statistical evaluation of the results was performed by the one-way ANOVA and Tukey test for multiple comparisons at the level of significance $p \leq 0.05$.

RESULTS AND DISCUSSION

The study was performed in the National Agricultural and Food Centre, Research Institute for Animal Production Nitra. Among the experimental groups no significant difference was noted in feed intake, feed conversion ratio and carcass value in the fattening experiment. Results regarding the zootechnical parameters are shown in Table 2. The study was divided into two phases: adaptation period (35^{th} day to 49^{th} day of age) and experimental period (49^{th} day to 91^{st} day of age).

Daily weight gain from weaning (on 35^{th} day) reached 38.75 vs. 43.33 g daily. The second phase over the study period at slaughtering age 91^{st} day reached 31.34 vs. 33.0 g daily weight gain; it was influenced by inorganic or organic sources of zinc supplementation in the rabbits' diet in groups 1EG and 2EG was compared with CG and 3EG. Increase in average body weight gain (from 0.89 to 1.24 g) was noted in the rabbits of group 1EG and 2EG compared to CG.

The average carcass dressing out percentage (58.37 vs. 60.12 %) was calculated. Results of selected meat quality parameters (content of water, content of proteins, fat and amino acids, fatty acids, electric conductivity, pH and colour) are presented in Table 3. Zinc supplementation raises levels of cholesterol, water holding capacity and energy value, and lowers the value of pH in *longissimus dorsi* muscle compared to rabbits fed the control diet. The fatty acids composition in MLD muscles is shown in Table 3. The rabbit muscles are a low-fat meat. The intramuscular lipid was characterized by the highest percentage of monounsaturated fatty acids (MUFA) (50.17 vs. 52.56 %). In this study the intramuscular lipids in the MLD muscles were also characterized by a higher percentage of saturated (SFA) (39.36 vs. 39.93 %) and lower percentage of polyunsaturated fatty acids (PUFA) (10.94 vs. 11.37 %). The higher percentage of MUFA in the intramuscular fats was determined in fine trial. The amino acid composition of MLD muscles is shown in Table 4. Rabbit protein contained a high amount of lysine, leucine, arginine, isoleucine, histidine, valine, threonine, phenylalanine, methionine and cystine in decreasing amounts. Moreover, the sequence of other amino acids is similar to the sequence of amino acids in other meats. The essential amino acid composition is one of the most important nutritional qualities of protein. The highest content of lysine, leucine, valine, threonine and sum of essential amino acids was specified in group 2EG with supplementation of Glycinoplex-Zn ($p \leq 0.05$). Nowadays, histidine is considered to be an essential amino acid because of the detrimental effects on haemoglobin concentrations (Report of a Joint WHO/ FAO/ UNU Expert Consultation, 2007). According to all of the detected amino acid scores, the protein in MLD muscle was well-balanced in essential amino acid composition and is of high quality. According to this study, lean rabbit meat could be considered a high quality protein source due to its well-balanced essential amino acid composition. No recent trials on the zinc requirements of rabbits could be traced in the literature, but levels of use vary between 25 and 60 $mg.kg^{-1}$, with the higher values proposed for does and bucks. Practical commercial diets contain a wider range of zinc (40–140 $mg.kg^{-1}$). Zinc oxide is the most commonly used source because it is less reactive and has a higher zinc concentration than sulphate and carbonate salts. No differences in zinc bioavailability between inorganic and organic sources have been reported in rabbits (Guimaraes and Motta, 2000; De Blas and Wiseman, 2010).

Because of zinc's environmental impact, the maximum level allowed in the EU for rabbit feeds is 150 mg.kg^{-1}. In addition, the adverse effect of high zinc intake on copper availability has to be considered (Maret and Sandstead, 2006). We agree with the proposal of EU for the Zn level in rabbits' diet, although in organic form of Zn we suggest to decrease the level in diet by about 20 %.

Table 3: The effect of dietary zinc supplementation on selected physico-chemical characteristics of MLD muscles 24 h *post mortem* ($\bar{x} \pm$ SD)

Characteristics (n = 6)	Control-C	1EG - with $ZnSO_4.H_2O$	2EG - with Glycinoplex- Zn	3EG - with Bioplex- Zn
Water g.100.g^{-1}	74.61 ± 0.44	74.89 ± 0.19	74.74 ± 0.55	74.64 ± 0.44
Protein g.100.g^{-1}	23.49 ± 0.43	23.31 ± 0.19	23.51 ± 0.27	23.67±0.57
Fat g.100.g^{-1}	0.92 ± 0.23	1.04 ± 0.13	0.89 ± 0.12	0.91 ± 0.21
Colour L	53.54 ± 1.94	52.21 ± 3.09	50.25 ± 2.98	48.88 ± 2.54
Electrical conductivity	1.41 ± 0.75	1.08 ± 0.40	2.50 ± 1.33	1.73 ± 0.85
Cholesterol g.100.g^{-1}	0.25 ± 0.06	0.30 ± 0.05^{a}	0.26 ± 0.05	0.27 ± 0.08
pH_{24}	6.16 ± 0.06	6.09 ± 0.07	6.07 ± 0.08	6.09 ± 0.06
Water holding capacity	26.33 ± 3.84	29.60 ± 3.08	31.03 ± 4.06	30.77 ± 3.52
EV(kJ.100 g^{-1})	427.15 ± 5.68	429.65 ± 4.55	427.30 ± 8.82	430.79 ± 8.69
	Fatty acids (% of total FA)			
Lauric (C12:0)	0.05 ± 0.007	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.01
Myristic (C14:0)	1.24 ± 0.04	1.26 ± 0.04	1.24 ± 0.03	1.24 ± 0.03
Palmitic (C16:0)	24.33 ± 0.24	24.60 ± 0.09^{ad}	24.44 ± 0.16	24.33 ± 0.12
Margaric (C17:0)	0.34 ± 0.02	0.34 ± 0.03	0.33 ± 0.01	0.34 ± 0.01
Stearic (C18:0)	11.26 ± 0.17	11.41 ± 0.22	11.45 ± 0.20	11.27 ± 0.28
Vaccenic (C18:1n9t)	4.41 ± 0.09	4.42 ± 0.06^{cd}	4.38 ± 0.05	4.43 ± 0.17
Oleic (C18:1n9c)	39.45 ± 1.63	40.65 ± 0.67^{a}	40.21 ± 1.53	40.44 ± 2.52
Linolic (C18:2n6c)	0.05 ± 0.00	0.05 ± 0.00	0.05 ± 0.00	0.05 ± 0.00
Linolenic (C18:3n3)	0.27 ± 0.02^{d}	0.28 ± 0.02^{d}	0.27 ± 0.04^{d}	0.26 ± 0.02
Eicosenoic (C20:1n^{-11})	0.50 ± 0.04	0.55 ± 0.04^{a}	0.52 ± 0.03^{a}	0.54 ± 0.04^{ac}
Eicosapentaenoic (C20:5n^{-3})	0.10 ± 0.01	0.10 ± 0.01	0.10 ± 0.01	0.11 ± 0.01
Arachidonic (C20:4n^{-6})	1.48 ± 0.22	1.52 ± 0.13	1.60 ± 0.15^{a}	1.60 ± 0.18^{a}
Docosapentaenic (C22:5n^{-6})	0.14 ± 0.00	0.14 ± 0.01	0.14 ± 0.01	0.15 ± 0.01
Docosahexaenic (C22:6n^{-3})	0.04 ± 0.00	0.045 ± 0.01	0.04 ± 0.01	0.04 ± 0.01
SFA	39.80 ± 1.23	39.93 ± 1.05	39.39 ± 0.87	39.36 ± 1.43
MUFA	51.36 ± 1.93	51.83 ± 1.86	52.56 ± 1.30^{d}	50.17 ± 3.51
PUFA	11.29 ± 1.42^{b}	10.94 ± 0.61	11.19 ± 0.32	11.37 ± 1.50^{bc}
PUFA/ SFA	0.28 ± 0.04	0.28 ± 0.02	0.28 ± 0.01	0.29 ± 0.01
ω3	0.60 ± 0.05	0.57 ± 0.09	0.56 ± 0.05	0.56 ± 0.09
ω6	10.33 ± 1.41^{b}	9.84 ± 0.48	10.40 ± 0.46^{b}	10.64 ± 1.57^{bc}
CLA	0.13 ± 0.02	0.13 ± 0.01	0.13 ± 0.01	0.14 ± 0.01

$^{a\,b\,c\,d}$ = values within the same different superscripts differ significantly ($p \leq 0.05$)

Table 4: The essential amino acid composition of MLD muscles (g.100.g^{-1})

Characteristics (n = 6)	Control-C	1EG - with $ZnSO_4.H_2O$	2EG - with Glycinoplex Zn	3EG - with Bioplex Zn
	$\bar{x}$ ± SD	$\bar{x}$ ± SD	$\bar{x}$ ± SD	$\bar{x}$ ± SD
Threonine	1.18 ± 0.05	1.15 ± 0.03	1.20 ± 0.08^{b}	1.16 ± 0.09
Valine	1.10 ± 0.04	1.10 ± 0.02	1.12 ± 0.07^{abd}	1.10 ± 0.08
Methionine	0.85 ± 0.02^{b}	0.82 ± 0.01	0.84 ± 0.05	0.83 ± 0.06
Cystine	0.37 ± 0.01	0.37 ± 0.01	0.37 ± 0.02	0.36 ± 0.02
Isoleucine	1.03 ± 0.04	1.02 ± 0.02	1.04 ± 0.07	1.02 ± 0.09
Leucine	2.11 ± 0.08	2.09 ± 0.06	2.14 ± 0.13^{bd}	2.08 ± 0.20
Phenylalanine	1.09 ± 0.03	1.07 ± 0.03	1.10 ± 0.07	1.07 ± 0.09
Histidine	1.26 ± 0.06	1.25 ± 0.06	1.27 ± 0.08	1.25 ± 0.14
Lysine	2.26 ± 0.08	2.03 ± 0.07	2.28 ± 0.15^{b}	2.23 ± 0.21
Arginine	1.68 ± 0.06	1.66 ± 0.05	1.70 ± 0.11	1.66 ± 0.16
Σ EAA	12.92 ± 0.48	12.80 ± 0.32	13.06 ± 0.83	12.77 ± 1.17

$^{a\,b\,c\,d}$ = values within the same different superscripts differ significantly ($p \leq 0.05$)

CONCLUSION

The growth rate in all groups was independent of zinc treatment. There was no statistically significant difference between the experimental and control groups in parameters of growth performance and the carcass yield.

A non-positive influence of zinc, dose of 38.46 g Glycinoplex-Zn and 66.67 g Bioplex-Zn per 100 kg of feed on animal health raises levels of cholesterol, water holding capacity and energy value, and lowers the value of pH, was noted in *longissimus dorsi* muscle compared to rabbits fed the control diet. Supplementation of Glycinoplex-Zn (100 mg of zinc) evoked a 25 % mortality rate with frequent *Pasteurella* infections to 8.3 % of the Bioplex-Zn (100 mg of zinc) as is shown in Table 2. These conditions were not observed in rabbits fed with other diets. The fatty acids content investigated in this study has proven statistically insignificant changes ($p \leq 0.05$). The highest content of lysine, leucine, valine, threonine and sum of essential amino acids was specified in group 2EG with supplementation of Glycinplex-Zn ($p \leq 0.05$). Results of this study suggest that lean rabbit meat could be a high quality protein source due to its well-balanced essential amino acids composition. Obtained results indicate to the prerequisite for inclusion of other additives in feed for breeding rabbits.

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EVALUATION OF *SACCHAROMYCES CEREVISIAE* SUPPLEMENTED POMEGRANATE SEED USING *IN VITRO* GAS PRODUCTION TECHNIQUE

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ABSTRACT

The aim of this study was to determine the chemical composition of pomegranate seed including tannin content, and gas production characteristics using *in vitro* gas production technique. The treatment contained 0, 2.5, 5 and 7.5 g yeast *Saccharomyces cerevisiae* (Sc) per kg of pomegranate seed based on DM (dry matter), respectively. CP, ADF, NDF, EE, ASH, TP (total phenolics) and TT (total tannin) contents in pomegranate seed were 12.2 %, 44.6 %, 62.3 %, 1.6 %, 12.1 %, 1.8 % and 0.8 %, respectively. At the early incubation time (2 h), the treatments 1 and 4 (treatment with Sc, 0 and 7.5 $g.kg^{-1}$ DM, respectively) had the highest gas production volume among treatments, but after 4 h incubation the gas production volume in treatments 1 and 4 was the lowest ($p < 0.05$). The treatment 2 at the most incubation times had the highest gas production volume. It may be concluded that *in vitro* gas production parameters of pomegranate seed was improved with addition of *Saccharomyces cerevisiae* at 2.5 $g.kg^{-1}$ DM.

Key words: *in vitro* gas production; pomegranate seed; *Saccharomyces cerevisiae*

INTRODUCTION

In Middle East, animals suffer from under feeding and malnutrition in winter due to the shortage of locally produced feeds which are not sufficient to cover the nutritional requirements of animals. A major constraint to increasing livestock productivity in developing countries is the scarcity and fluctuating quantity and quality of the year-round supply of conventional feeds. These countries experience serious shortages in animal feeds of the conventional type. In order to meet the projected high demand of livestock products and to fulfil the future hopes of feeding the millions and safeguarding their food security, the better utilization of non-conventional feed resources which do not compete with human food is imperative. There is also a need to identify and introduce new and lesser known food and feed crops. An important class of non-conventional feeds is by-product feedstuffs which are obtained during harvesting or processing of a commodity in which human food or fibre is derived. The amount of by-product feedstuffs generally increases as the human population increases and economies grow (Besharati *et al.*, 2008; Besharati and Taghizadeh, 2009; Besharati and Taghizadeh, 2011). Increasing agricultural industrial units for producing pomegranate juice leads to the accumulation of pomegranate peel and the annual production of this by-product is approximately 120.000 metric tons in Iran (Mirzaei-Aghsaghali *et al.*, 2011). Pomegranate fruit consists of three parts: the seeds, the juice and the peels which include the husk and interior network membranes (Shabtay *et al.*, 2008).

Several factors have led to increased interest in by-product feedstuffs, such as pollution abatement and regulations, increasing costs of waste disposal and changes in perception of the value of by-product feedstuffs as economical feed alternatives (Besharati *et al*., 2008; Besharati and Taghizadeh, 2009; Besharati and Taghizadeh, 2011).

Probiotics present an attractive alternative to the use of chemical and hormonal promoters in the livestock growth production industry. The preparations

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have been used for production of safe by micro-organisms for many years and thus are generally accepted in food by both the farmer and the final consumer. *Saccharomyces cerevisiae* supplementation in ruminant diets can increase DMI, production performance, cellulose degradation, and nutrient digestibility (Callaway and Martin, 1997). The gas measuring technique has been widely used for evaluation of nutritive value of feeds. Gas measurement provides a useful data on digestion kinetics of both soluble and insoluble fractions of feedstuffs (Getachew *et al.*, 1998). In the gas method, kinetics of fermentation can be studied on a single sample and therefore a relatively small amount of sample is required or a larger number of samples can be evaluated at a time. Besharati *et al*. (2009) showed that probiotics can improve the *in vitro* gas production. The purpose of this study was to study the effect of adding different levels of *Saccharomyces cerevisiae* on *in vitro* gas production of biscuit by-product.

There is a little information available regarding the nutritive value of pomegranate seed (PS) produced in Iran. The aim of this study was to determine the chemical composition including tannin content of pomegranate seed supplemented with *Saccharomyces cerevisiae* and gas production characteristics using *in vitro* gas production technique.

MATERIAL AND METHODS

Pomegranate seed

Pomegranate seed was obtained from a fruit juice manufacturing factory in Tabriz, Iran.

Chemical composition

Pomegranate seed dry matter (DM, method ID 934.01), ash (method ID 942.05), ether extract (EE, method ID 920.30) and crude protein (CP, method ID 984.13) were determined by standard procedures (AOAC, 1999). The NDF and ADF concentrations were determined using the methods of Van Soest *et al.* (1991) without sodium sulphite. NDF was analysed without amylase with ash included.

Total phenolics (TP) were measured using the Folin Ciocalteau method (Makkar, 2000). Total tannin (TT) was determined after adding insoluble polyvinylpyrrolidone and reacting with Folin Ciocalteau reagent (Makkar, 2000). Tannic acid was used as the standard to express the amount of TP and TT.

In vitro gas production trial

The dry matter degradability of each by-product was determined by *in vitro* fermentation with ruminal fluid. Ruminal fluid was collected approximately 2 h after morning feeding from two cannulated sheep consuming 400 g alfalfa hay, 300 g barley and 300 g soybean meal. Ruminal fluid was immediately squeezed through four layers of cheesecloth and was transported to the laboratory in a sealed thermos. The resulting ruminal fluid was purged with deoxygenated CO_2 before use as the inoculum. Gas production was measured by Fedorak and Hurdey (1983) method. The treatment contained 0, 2.5, 5 and 7.5 g *Saccharomyces cerevisiae* (Sc) per kg of pomegranate seed based on DM, respectively. Approximately 300 mg of dried and ground (2 mm) pomegranate seeds were weighed and placed into serum bottles. There were 3 replicates per treatment. Buffered rumen fluid with McDougal buffer (20 ml) was pipetted into each serum bottle (McDougall, 1948). The gas production was recorded after 2, 4, 6, 8, 12, 16, 24, 36, and 48 h of incubation. Total gas values were corrected for the blank incubation, and reported gas values are expressed in ml per 1 g of DM. The metabolizable energy (ME) contents of treatments and OMD were calculated using equations of Menke *et al.* (1979) as:

$$ME_{(MJ\,kg^{-1}\,DM)} = 2.20 + 0.136 \times GP + 0.057 \times CP + 0.0029 \times CP^2$$

$$OMD_{(g\,100\,g^{-1}\,DM)} = 14.88 + 0.889 \times GP + 0.45 \times CP + 0.0651 \times XA$$

where XA ash is in g 100 g^{-1} DM and GP is the net gas production (ml) at 24 h. The short chain fatty acids were calculated using blow equation as:

$$SCFA_{(mmol)} = -0.00425 + 0.0222GP$$

where Gas is 24 h net gas production (ml 0.2 g^{-1} DM).

Statistical analysis

Data obtained from *in vitro* gas production study was subjected to analysis of variance as a completely randomized design by the GLM procedure of SAS Institute Inc (2002) and treatment means were compared by the Duncan test.

RESULTS AND DISCUSSION

The chemical compositions of pomegranate seeds are shown in Table 1. CP, ADF, NDF, EE, ASH, TP and TT contents in pomegranate seeds were 12.2 %, 44.6 %, 62.3 %, 1.6 %, 12.1 %, 1.8 % and 0.8 %, respectively. Chemical compositions of pomegranate seeds in the current study were inconsistent with findings of Taher-Maddah *et al*. (2012). Feizi *et al.* (2005) reported that DM, OM, CP, crude fibre, and EE values of pomegranate seeds were 94.8, 96.8, 11.4, 38.9, and 1.0 %, respectively. These differences in chemical composition of by-products may be due to a difference in cultivar, growing conditions, varieties, and different de-hulling processes (Taher-Maddah *et al*., 2012). Kamalak *et al.* (2007) reported that total and soluble condensed tannins, NDF and ADF were negatively

correlated with estimated parameters of gas production. The results of our study are consistent with those of Feizi *et al.* (2005) who suggested that pomegranate peel tannins have negative effect on *in vitro* rumen fermentation. Tannins are considered to have both adverse and beneficial effects in ruminant animals. High concentrations of tannins may reduce intake, digestibility of protein and carbohydrates, and animal performance through their negative effect on palatability and digestion. By preventing bloat and increasing the flow of non-ammonia nitrogen and essential amino acids from the rumen, low and moderate (20-45 mg/g DM) concentrations of condensed tannins in the diet improved production efficiency in ruminants, without increasing feed intake (Shabtay *et al.*, 2008). In the last few years there is an increasing interest of nutritionists in bioactive plant factors - phytofactors as natural feed additives, tannins etc. that can modify the rumen fermentation processes (e.g. defaunation), improve the protein metabolism and, at the same time, reduce ammonia production and emission, and curb methane production and emission to the atmosphere. High diversity of bioactive phytofactors contained in many plant species has been identified as a potential factor affecting the above-mentioned processes (Szumacher-Strabel and Cieślak, 2010).

Table 1: Chemical composition of pomegranate seeds (% of DM)

	Components							
	DM	CP	EE	NDF	ADF	ASH	TP	TT
Pomegranate seed	93.8	12.2	1.6	62.3	44.6	12.1	1.8	0.8

DM = Dry matter; CP = Crude protein; EE = Ether extract; ADF = Acid detergent fibre; NDF = Neutral detergent fibre; TP = Total phenol; TT = Total tannins

Gas production volumes (ml.g^{-1} DM) from *in vitro* incubation of PS supplemented with different levels of *Saccharomyces cerevisiae* at different incubation times are shown in Table 2 and Fig. 1. The cumulative volume of gas production increased with increasing time of incubation. Although there are other models available to describe the kinetics of gas production, the Ørskov and McDonald (1979) method was chosen because the relationship of its parameters with intake, digestibility and degradation characteristic of forages and concentrate feedstuffs had been documented. Sommart *et al.* (2000) reported that gas volume is a good parameter for predicting digestibility, fermentation end product and microbial protein synthesis of the substrate by rumen microbes in the *in vitro* system. Gas volumes have also showed a close relationship with feed intake (Blummel and Becker, 1997) and growth rate in cattle (Blummel and Ørskov, 1993).

At the early incubation time (2 h), the treatments 1 and 4 (treatment with Sc, 0 and 7.5 ml.g^{-1} DM,

Table 2: Total gas production volume (ml/g DM) in incubation times

Treatments	Incubation times (h)								
	2	4	6	8	12	16	24	36	48
Pomegranate seed	31.52^{a}	33.45^{c}	38.11^{b}	57.42^{c}	82.51^{ab}	87.79^{bc}	95.11^{b}	106.50^{c}	109.47^{c}
PS + Sc 2.5 g.kg^{-1} DM	21.31^{b}	46.22^{a}	65.86^{a}	82.06^{a}	96.42^{a}	106.14^{a}	121.68^{a}	138.73^{a}	145.25^{a}
PS + Sc 5 g.kg^{-1} DM	21.98^{b}	43.99^{ab}	62.42^{a}	74.85^{ab}	88.76^{ab}	98.93^{ab}	112.25^{a}	127.18^{b}	135.04^{b}
PS + Sc 7.5 g.kg^{-1} DM	33.08^{a}	36.45^{bc}	45.99^{b}	64.97^{bc}	75.05^{b}	81.86^{b}	98.28^{b}	111.33^{c}	115.86^{c}
SEM	2.84	2.85	3.10	3.57	4.22	4.27	3.81	2.52	2.43

The means within a column without common letters differ ($p < 0.05$).

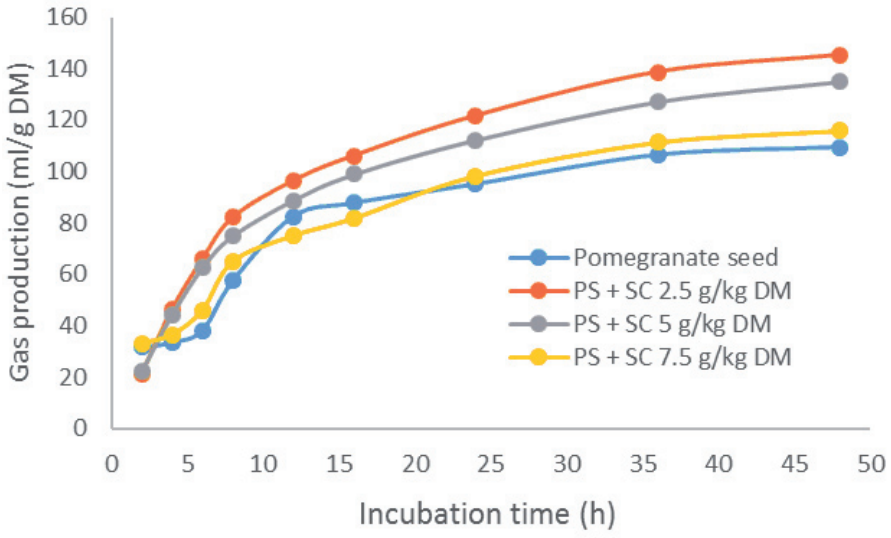

Fig. 1: Pattern of *in vitro* gas production (fitted with exponential model) as affected by different levels of SC at different incubation times

respectively) had the highest gas production volume among treatments, but after 4 h incubation the gas production volume in treatments 1 and 4 was the lowest ($p < 0.05$). The treatment 2 at the most incubation times had the highest gas production volume.

Mirzaei-Aghsaghali *et al*. (2011) showed that the gas production after 48 h incubation for PS was 222.3 $ml.g^{-1}$ DM, which was higher than the amount obtained in this study (109.47 $ml.g^{-1}$ DM).

These results are in agreement with a previous study that *Saccharomyces cerevisiae* increases ruminal gas production (Martin and Nisbet, 1992), but others found no effect (Lila *et al*., 2004) or a decrease (Lynch and Martin, 2002) in batch cultures with mixed rumen microflora. The discrepancies among studies could be associated with the characteristics of the strain, diet composition (Sullivan and Martin, 1999) and dose (Lila *et al*., 2006).

The ability of yeast to increase IVGP observed in the study has been reported by various authors with different roughages (Chaucheyras-Durand *et al*., 2008; Ando *et al.*, 2004; Ando *et al*., 2005). Tang *et al*. (2008) reported an increase in rate of gas production and IVRDMD (*in vitro* rumen dry matter digestibility) from yeast supplementation of low quality cereal straws that was associated with an increase in protozoa and cellulolytic bacteria populations. Increase in bacterial population and activity of rumen microbes that led to higher IVRDMD as a result of yeast supplementation may be attributed to ability of yeast to remove oxygen from the rumen environment and to effects of organic acids, essential enzymes and vitamins derived from yeast activity or yeast components themselves such as peptides and amino acids (Fonty and Chaucheyras-Durand, 2006; Ding, 2008). Kim *et al.* (2005) reported a significant positive correlation between ruminal molar proportions of branched-chain fatty acids (BCFA) and the efficiency of microbial protein synthesis. The BCFA are required for resynthesis of branched-chain amino acids for microbial protein synthesis in the rumen (Allison, 1969). An *in vitro* fermentation study demonstrated that BCFA supplementation could increase microbial protein synthesis and DM digestion (Cummins and Papas, 1985). It is assumed that true protein supplementation via yeast could have been beneficial for BCFA production in the process of protein degradation in the rumen and consequently resulted in a greater increase in IVRDMD for Japanese sake yeast and bio ethanol residue yeast as compared with soybean peptide (SP).

Wambui *et al*. (2010) used two strains of *Sacharomyces cereviciae* (Japanese sake yeast and bio ethanol residue yeast). Both Japanese sake yeast (JSY) and bio ethanol residue yeast supplements increased the ruminal digestion of the browse foliages and the effect of JSY appeared to be significantly higher. Differences in effect of yeast on rumen microbes and fermentation patterns are mainly associated with the strain of Sacharomyces cereviciae used (Ando *et al*., 2005). Certain strains of yeast are more effective

at stimulating certain groups of bacteria and ruminal fermentation than others. Ability of yeast to influence rumen fermantation is more pronounced when live yeast cells are used as opposed to autoclaved yeast cultures or yeast derivatives (Ando *et al*., 2005; Wambui *et al.*, 2010).

Ando *et al*. (2005) also pointed out that the differences in the yeasts' metabolic functions or cell wall structures can influence their degradability of roughages. Efficacy of yeast products on rumen fermentation and animal performance is also greatly influenced by the diet (Chaucheyras-Durand *et al*., 2008). It is postulated that factors such as the structure and biological activity of tannins and presence of other antinutritive compounds may have influenced the results observed. Further studies on the effect of yeast supplementation on the nitrogen (N) degradation in the rumen and a subsequent effect on post-ruminal N digestion status are needed.

Estimated gas production parameters of treatments are shown in Table 3. The treatment with 2.5 SC g.kg^{-1} DM had the highest ME, OMD and SCFA among treatments.

Table 3: Estimated gas production parameters of treatments

Treatments	Items			
	GP $_{(ml\ 0.2\ g^{-1}\ DM)}$	ME $_{(MJ.kg^{-1}\ DM)}$	OMD $_{(g\ 100\ g^{-1}\ DM)}$	SCFA $_{(mmol)}$
Pomegranate seed	19.02^{b}	5.91^{b}	38.07^{b}	0.418^{b}
PS + Sc 2.5 g.kg^{-1} DM	24.34^{a}	6.64^{a}	42.79^{a}	0.536^{a}
PS + Sc 5 g.kg^{-1} DM	22.45^{a}	6.38^{a}	41.12^{a}	0.494^{a}
PS + Sc 7.5 g.kg^{-1} DM	19.66^{b}	6.00^{b}	38.63^{b}	0.432^{b}
SEM	0.491	0.430	0.135	0.0101

ME = Metabolizable energy (MJ.kg^{-1} DM); OMD = Organic matter digestibility (% DM); SCFA = Short chain fatty acid (mmoL).
The means within a column without common letters differ ($p < 0.05$).

CONCLUSION

It was concluded that *in vitro* gas production parameters of pomegranate seed was improved with addition of *Saccharomyces cerevisiae* at 2.5 g.kg^{-1} DM level.

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SUPPLEMENT OF SODIUM BICARBONATE, CALCIUM CARBONATE AND RICE STRAW IN LACTATING DAIRY COWS FED PINEAPPLE PEEL AS MAIN ROUGHAGE

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ABSTRACT

The aim of this study was to evaluate the effect of buffering agents on performance of lactating cows fed pineapple peel as main roughage. Four mid-lactation primiparous crossbred Holstein dairy cows averaging 443.5 ± 10.6 kg BW were assigned in a 4 x 4 Latin square design. Each cow was fed one of four experimental diets including: T1) control, pineapple peel (PP) to commercial pellet (CL) ratio of 70:30 without added buffer; T2) PP to CL ratio of 70:30 with 1.2 % sodium bicarbonate ($NaHCO_3$); T3) PP to CL ratio of 52.5: 30 with mixture of 17.5 % rice bran and 1.2 % calcium carbonate ($CaCO_3$); and T4) PP to CL ratio of 50:30 with 20 % rice straw (RS). The results revealed that feed intake, digestion coefficient, digestible nutrient intake were unaffected by supplementation of $NaHCO_3$, $CaCO_3$, and RS in diets ($P > 0.05$). The daily quantities of ME and NEL intake were not altered by treatments ($P > 0.05$), but PDIE and PDIN were increased by supplementing $CaCO_3$ in the diet ($P < 0.05$). Weight gain was higher for cows supplemented with $NaHCO_3$ and RS compared with other groups ($P < 0.05$). Cows receiving supplemental $NaHCO_3$, $CaCO_3$ and RS had the same concentration of volatile fatty acids ($P > 0.05$). Acetate to propionate ratio ranged between 2.18 to 2.96 ($P > 0.05$) with the highest (2.96) in the RS supplement group. The $NaHCO_3$, $CaCO_3$, and RS supplement did not influence blood metabolites, blood electrolytes, milk yield and milk composition ($P > 0.05$). No sign of acidosis was observed. Therefore, it could be concluded that $NaHCO_3$, $CaCO_3$, or RS supplementation had no significant impact on performance of lactating cows fed PP as main roughage. Further research should be conducted to test the influence of such diets on milk production with larger number of animals in longer period of time.

Key words: sodium bicarbonate; calcium carbonate; rice straw; pineapple peel; dairy cow

INTRODUCTION

Local feed resources and agricultural by-products are of prime importance for ruminants raised in the tropics (Wanapat, 2000). Pineapple peel is a cannery by-product of Pineapple (*Ananas comosus*), a tropical fruit which largely grows in Brazil, Thailand, Philippines, China and several other countries (FAO, 2013). Pineapple peel is a potential roughage source for ruminants due to the large amount of effective fiber and some sugars (Datt *et al*., 2008; Paengkoum *et al*., 2013; Nadzirah *et al*., 2013) which can be used by rumen microbes to digest and synthesize for animal energy supply as well as lactose synthesis in mammary gland (Russell, 2002). The nutrients in pineapple peel consists of dry matter (DM), organic matter (OM), crude protein (CP), neutral detergent fibre (NDF), acid detergent fibre (ADF) and ash which are 12.6, 88.6, 8.7, 67.7, 50.3 and 11.4, respectively (Paengkoum *et al*., 2013). However, chemical property of pineapple peel is rather low in pH (3.47-3.84) (Nadzirah *et al.*, 2013) which may affect rumen ecology and productive performance if large amount of pineapple peel is being fed to dairy cows. Feeding diets high in nonstructural

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carbohydrates or acid load usually decreases ruminal pH and may cause ruminal acidosis (Owens *et al*., 1998; Rustomo *et al*., 2006). In clinical acidosis, cows will suffer from rumenitis, metabolic acidosis, lameness, hepatic abscessation, pneumonia and death while those in subclinical acidosis will lower feed intake, lower feed digestibility and subsequent lower milk fat content (Lean *et al*., 2000).

Sodium bicarbonate ($NaHCO_3$) is one of dietary buffer commonly used to prevent ruminal pH reduction and enhance ruminal fermentation in low roughage diet (Le Ruyet and Tucker, 1992; Russell and Chow, 1993). NRC (1989) suggested that $NaHCO_3$ should be added 1.2 – 1.6 % in concentrate mixture to control ruminal pH when diets were high in nonstructural carbohydrates or acids. Calcium carbonate ($CaCO_3$) or limestone is locally available buffer. However, $CaCO_3$ has little or no buffering effect when the rumen pH is 6.0 or above because of its low solubility in ruminal fluid at pH above 5.5 (Clark *et al*., 1989). Rice straw (RS) is a local agricultural by product which is abundant in effective fibre which promote chewing activity and saliva secretion. Saliva contains $NaHCO_3$ which acts as a buffer to control ruminal pH in ruminants (Russell and Chow, 1993). The available data involved in PP feeding to lactating dairy cows concerning the incident of rumen acidosis is limited. Our study was conducted to evaluate whether feeding PP supplemented with $NaHCO_3$, $CaCO_3$ and RS would reduce risk of subclinical acidosis as measured by feed intake and digestible nutrient intake variation, ruminal fermentation, blood metabolites, blood electrolytes, milk production and milk composition.

MATERIAL AND METHODS

Animals, Experimental Design and Diet

Four primiparous, midlactation (84 ± 18 d in milk) crossbred Holstein cows (n = 4) initially averaging 443.5 ± 10.6 kg body weight (BW) were assigned with four successive periods in 4 x 4 Latin square design. Each 21-d experimental period consisted of 14-d for animal adaptation to the diet and 7-d for sample and data collection. Treatments consisted of: T1) control diet, pineapple peel (PP) to commercial pellet (CL) (Charoen Pokphand PCL, Thailand) ratio of 70:30 on dry matter basis without added buffer, T2) PP to CL ratio of 70:30 with 1.2 % $NaHCO_3$, T3) PP to CL ratio of 52.5: 30 with mixture of 17.5 % rice bran (RB) and 1.2 % $CaCO_3$, and T4) PP to CL ratio of 50:30 with 20 % rice straw (RS) (Table 1). Feed for each cow was balanced depending on its body weight, milk yield and milk fat following the recommended nutrient requirement as stated by NRC (2001). All diets were formulated to support nutrient need for maintenance and lactation of cows approximately 63.49 ± 1.95 $MJ.d^{-1}$ of NEL and 1.17 ± 0.05 $kg.d^{-1}$ of dietary CP concentration (Table 1). Each feed ingredient was weighed individually before distribution as a mixed feed.

The PP using in this experiment was collected from a cannery factory in Kanchanaburi province in the west of Thailand, stored approximately 30 kg each in a sealed double layer polyethylene plastic bag without any preservative agents. In T1 and T2, PP was used as the roughage and energy sources, whereas in T3 and T4 a reduction in PP was replaced with RB and RS, respectively. RB was added in T3 to increase palatability and it was a locally available feed. However, RB was high in fat content. RS was added in T4 to stimulate chewing activity to increase saliva secretion. Each lactating dairy cow was housed individually in a 3.0 x 6.0 m^2 pen, in which drinking water and mineral blocks were available throughout. Cows were fed twice daily at 07:00 h and 17:00 h at 110 % of expected intake throughout the experiment. Cows were moved to milking parlour and milked twice daily at 06:00 and 15:00 h. Animal management and experimental protocol was performed with respect to animal care and welfare.

Measurement, Sample Collection and Analyses

Feed offered and refused were recorded daily in all last 7-d of each data collection period. In the first 7-d of each adaptation period, PP and CL were collected and dried in a 60 °C hot air oven for 72 h for DM concentration determination in order to correct daily feed intake. All cows were weighed three times (d1, d14 and d21) during each period to calculate and predict feed intake. Regular feed samples from individual cows were collected during the last 7-d of each period. Then, feed samples were dried at 60 °C for 72 h; ground and composited and analyzed for chemical composition including DM, CP, EE, ash, Ca and P by the method of AOAC (1984). Neutral detergent fibre (NDF) and acid detergent fibre (ADF) were measured by the method of Goering and Van Soest (1970). Acid insoluble ash (AIA) as a natural marker in feed was measured by the method of Van Keulen and Young (1977).

During the last 5-d of each data collection period, fecal grab samples were collected twice daily at 12 h intervals, pooled on an equal wet-weight basis for each cows, dried at 60 °C for 72 h, ground and analyzed for DM and CP by the method of AOAC (1984), NDF and ADF by the method of Goering and Van Soest (1970) and AIA by the method of Van Keulen and Young (1977). Digestibility coefficients of nutrients were calculated using equations given by Schneider and Flatt (1975): DM digestibility, % = 100 – [100 × (AIA % in feed) ÷ (AIA % in feces)]; Nutrient digestibility, % = 100 – [(100 × AIA % in feed ÷ AIA % in feces) x (nutrient % in feces ÷ nutrient % in feed)]. Organic matter (OM) or the loss

Table 1: Ingredients, chemical composition of diets and daily nutrient requirement of dairy cows

Items					Complete feed Mixtures			
					T1	T2	T3	T4
Ingredients, kg.100 kg DM^{-1}								
PP					70.00	69.16	51.87	50
CL					30.00	29.64	29.64	30
RB					-	-	17.29	-
RS					-	-	-	20
$NaHCO_3$					-	1.2	-	-
$CaCO_3$					-	-	1.2	-
Total					100	100	100	100
Chemical composition, g.kg DM^{-1}								
	PP	CL	RB	RS	T1	T2	T3	T4
DM	340.96	958.50	960.10	949.60	929.45	918.29	925.74	935.97
OM	908.50	885.50	901.20	849.30	901.60	890.78	889.51	889.76
CP	73.80	187.80	142.00	44.30	108.00	106.70	118.49	102.10
EE	23.30	52.40	220.60	16.50	32.03	31.64	65.75	30.67
NDF	584.80	358.20	315.80	763.10	516.82	510.61	464.10	552.48
ADF	278.00	165.50	78.20	469.70	244.25	241.31	206.77	288.59
Ash	91.50	114.50	98.80	150.70	98.40	97.21	98.48	110.24
Ca	8.20	16.90	3.60	4.10	10.81	10.68	9.93	9.99
P	2.00	8.50	19.90	0.80	3.95	3.90	6.99	3.71
pH	3.55	-	-	-	-	-	-	-
Daily nutrient requirement					T1	T2	T3	T4
NEL, $MJ.d^{-1}$					61.88	62.84	62.92	66.35
NEL, MJ.kg DM^{-1}					5.81	5.94	5.85	6.06
CP, $kg.d^{-1}$					1.13	1.16	1.16	1.26
NDF, $kg.d^{-1}$					2.98	2.96	3.02	3.07
ADF, $kg.d^{-1}$					2.23	2.22	2.26	2.30
Ca, $g.d^{-1}$					47.26	48.22	48.3	52.10
P, $g.d^{-1}$					30.80	31.39	31.44	33.80

PP = pineapple peel, CL = commercial pellet, RB = rice bran, RS = rice straw, T1 = PP to CL ratio of 70:30, T2 = PP to CL ratio of 70:30 with 1.2 % $NaHCO_3$ supplement, T3 = PP to CL ratio of 52.5: 30 with supplement of 17.5 % RB and 1.2 % $CaCO_3$ mixture, T4 = PP to CL ratio of 50:30 with 20 % RS.

in DM weight after incubation at 550 ℃ for 15 h was calculated as follows: OM = 100 – Ash %.

Values for metabolizable energy (ME) were calculated by prediction from digestible organic matter intake (DOMI) as follows: 1 kg DOMI = 3.8 Mcal ME.kg DM^{-1} 4.184 (Kearl, 1982). Net energy of lactation (NEL) was estimated at actual intake when feed EE content was above 3 % by the equation: NEL (MJ.kg DM^{-1}) = 0.703 × ME – 0.19 + ([(0.097 × ME + 0.19) /97] × [EE–3]) × 4.184 (NRC, 2001). Values for feed protein truly digestible in the small intestine (PDIA); protein truly digestible in the small intestine where N is limiting microbial protein synthesis (PDIN); and protein truly digestible in the small intestine where energy is limiting microbial protein synthesis (PDIE) were calculated using the equations given by Jarrige (1989): PDIN (g.kg M^{-1}) = PDIA + [0.64 × CP(g.kg DM^{-1}) × (deg – 0.1)] where PDIA = CP(g.kg DM^{-1}) × 1.11(1 – deg) × dsi; PDIE = PDIA + DIME where PDIME (g.kg DM^{-1}) = 0.093 × [FOM – EE (g.kg DM^{-1})].

FOM is the fermentable organic matter content (g.kg DM^{-1}) (Jarrige, 1989; Beatriz Tobias *et. al.*, 2006). The deg value is theoretical degradability of feeds *in sacco* and dsi value is the true digestibility of undegraded dietary protein in the small intestine (Jarrige, 1989). Both deg and dsi were obtained from published data (Jarrige, 1989; Susmel *et. al.*, 1989; Pozdíšek *et. al*., 2003; Beatriz Tobias, *et. al.*, 2006).

Ruminal samples were taken by suction pump at 4 h post feeding and measured pH immediately by portable pH meter (pH Tester 30®, EUTECH Instruments, Singapore). The 50 ml of rumen fluid were filtered through four layers of cheesecloth, added with 5 mL of 6*N* H_2SO_4 to stop fermentation, centrifuged at 3,000 rpm for 10 minutes and kept supernatant frozen at –20 °C until later analyses for volatile fatty acid using an analytical High Performance Liquid Chromatography (HPLC, Agilent technologies 1100 series, Germany). 10 mL of blood samples were taken from coccygeal vein and subsequent analysis for glucose, urea nitrogen and electrolytes using enzymatic and kinetic methods (Synchron LXSystem/Lxi725, Beckman Coulter Inc.). Milk samples of each cows were collected during milking in the morning and afternoon at ratio of 60 to 40 for 4 consecutive days, composited and analyzed for fat, protein, lactose, solid not fat and total solid by Combi Foss 6000 (Foss Electric, Hillerød, Denmark).

Statistical Analysis

Data were analyzed using the general linear model procedure wherein treatment means were compared by Duncan's new multiple range test and significance was declared when P-value < 0.05 (SPSS, 2006). The statistical model used was $Y_{ij(k)} = \mu + \rho_i + \gamma_j + \tau_{(k)} + \varepsilon_{ij}$ where $Y_{ij(k)}$ = dependent variable, μ = overall mean, ρ_i = effect of period (i=1,2,3,4), γ_j = effect of animal (j = 1,2,3,4), $\tau_{(k)}$ = effect of treatment, and ε_{ij} = random error (Steel and Torrie, 1980).

RESULTS AND DISCUSSION

The chemical composition of ingredients and complete feed mixtures are presented in Table 1. The average DM content of PP was 340.96 g.kg DM^{-1} or 34.09 % DM. Previous studies (Datt *et al*., 2008); Suksathit *et al.*, 2011) reported that DM content of pineapple waste is 138.50-168.70 $g.kg^{-1}$. The higher DM content of PP in this experiment is due to the moisture loss before packing into double layer polyethylene plastic bags. Proper conservation such as storage in sealed plastic bags could prevent mold growth and help to control both nutritive value and palatability of PP throughout the experiment. The CL used in this study contained CP, NDF and ADF of 187.80, 358.20 and 165.50 g.kg DM^{-1}, respectively.

Total feed intake in all lactating cows was not affected by $NaHCO_3$, $CaCO_3$ or RS supplementation (P > 0.05) (Table 2). However, PP intake was significantly increased by $NaHCO_3$ supplementation and averaged 13.87 kg $DM.d^{-1}$ or 3.12 % BW/d (P<0.05) (Table 2). The increasing response in feed intake by $NaHCO_3$ supplement has been demonstrated in other trials (Rogers *et al*., 1985; Vicini *et al*., 1988). In contrast, a number of trials reported the lack of response to $NaHCO_3$ supplementation on feed intake (Erdman *et al*., 1982; Wittayakun *et al*., 2006 a,b; Doepel and Hayirli, 2011). Supplementation with $NaHCO_3$ may have had an effect on osmolality and pH in the rumen. Addition of $CaCO_3$ and RS in other groups did not affect PP intake (P > 0.05).

The digestion coefficient and digestible nutrient including OM, CP, NDF and ADF were not affected by addition of $NaHCO_3$, $CaCO_3$ or RS (P > 0.05) (Table 2). The physical form of PP containing high fiber content is also an important factor which may alter digestibility. Suksathit *et al.* (2011) reported that the use of pineapple waste as sole roughage source had a positive effect on digestibility when compared with hay. ME and NEL intake were not affected by treatments (P > 0.05). The ME intake averaged 11.16 ± 0.72 ranging from 10.81-11.54 MJ.kg DM^{-1}. The NEL intake averaged 7.06 ± 0.51 MJ.kg DM^{-1} or 117.77 ± 17.45 $MJ.d^{-1}$ which was higher than the nutrient requirement recommended by NRC (2001). The $CaCO_3$ supplement increased supply of PDIN and PDIE (P < 0.05). In this study, PDIN was always lower than PDIE (Table 2).

Influence of treatments on body weight, rumen pH, VFA concentration, blood metabolites, milk yield, and milk composition of dairy cows are shown in Table 3. Initial and final body weight of the cows were similar in all cows (P > 0.05). However, average daily weight change of those cows supplemented with $NaHCO_3$ and RS tended to increase more than those fed only PP or PP with $CaCO_3$ (P < 0.05). Those cows fed PP with $CaCO_3$ had significantly decreased body weights (P < 0.05). Changes in body weight may indicate efficiency of productive improvement. However, the influence of treatments on body weight may need longer time to verify.

The average ruminal pH across treatments was 6.78 ± 0.34. The average rumen pH was not significantly affected by $NaHCO_3$, $CaCO_3$ and RS supplementation (P > 0.05) (Table 3). However, $CaCO_3$ supplementation tended to increase ruminal pH when compared with other treatments (Table 3). Normally, $CaCO_3$ has

Table 2: Influence of treatments on intake, digestion coefficient, digestible nutrient intake and nutritive values

Items	T1	T2	T3	T4	SE	*P-value*
Total Feed Intake						
kg DM	15.29	17.55	17.79	15.79	1.27	0.076
% BW	3.43	3.95	3.95	3.50	0.27	0.068
PP Intake						
kg DM	11.61[a]	13.87[b]	10.85[a]	11.32[a]	1.14	0.037
% BW	2.61[a]	3.12[b]	2.40[a]	2.51[a]	0.24	0.026
Digestion coefficient, %						
DM	72.30	74.37	77.79	76.36	5.66	0.580
OM	75.36	76.95	80.46	79.18	5.05	0.532
CP	57.32	58.88	69.70	65.69	10.48	0.378
NDF	70.60	72.57	74.58	75.25	5.54	0.653
ADF	68.37	70.64	70.65	71.89	5.49	0.836
Digestible nutrient intake, kgDM/d						
DM	12.54	13.08	13.91	12.05	1.93	0.602
OM	10.43	12.23	12.98	11.16	1.63	0.229
CP	0.89	1.01	1.36	1.02	0.23	0.114
NDF	5.74	6.86	6.53	6.42	1.01	0.514
ADF	2.63	3.16	2.76	2.96	0.48	0.475
Nutritive value						
ME[1], MJ.kg DM^{-1}	10.81	11.05	11.54	11.23	0.72	0.580
NEL[2], MJ.kg DM^{-1}	6.80	6.97	7.36	7.09	0.51	0.530
NEL, MJ.d^{-1}	104.44	122.78	131.68	112.16	17.45	0.235
PDIN[3], g.kg DM^{-1}	76.95[a]	73.68[a]	83.66[b]	74.95[a]	2.23	0.003
PDIE[4], g.kg DM^{-1}	81.06[a]	78.09[a]	91.07[b]	79.50[a]	2.24	0.001

T1 = PP to CL ratio of 70:30, T2 = PP to CL ratio of 70:30 with 1.2 % $NaHCO_3$ supplement, T3 = PP to CL ratio of 52.5: 30 with supplement of 17.5 % RB and 1.2 % $CaCO_3$ mixture, T4 = PP to CL ratio of 50:30 with 20 % RS.
Within rows, means followed by different letters are significantly different at $P < 0.05$.
[1]1kg DOMI = 3.8 McalME.kg DM^{-1} × 4.184 (Kearl, 1982).
[2]NEL (Mcal.kg^{-1}) = 0.703 × ME – 0.19 + ([(0.097 × ME + 0.19)/97] × [EE – 3]) × 4.184 (NRC, 2001).
[3]PDIN = protein truly digested in the small intestine with nitrogen-limiting microbial protein synthesis in the rumen (Jarrige, 1989).
[4]PDIE = protein truly digested in the small intestine with energy-limiting microbial protein synthesis in the rumen (Jarrige, 1989).

no buffering effect when rumen pH is greater than 6.0 due to its low solubility (Rogers *et al*., 1985). Physical form of PP had thick and long particle size which contained 584.80 g.kg^{-1} NDF and 278.00 g.kg^{-1} ADF (Table 1). It may stimulate chewing activity and saliva secretion which may affect fluid dilution rate and pH control in the rumen. The $NaHCO_3$ in saliva is also an extra buffering agent involved in ruminal pH control which can act effectively when rumen pH is above 5.7 (Russell, 2002).

The supplementation of $NaHCO_3$, $CaCO_3$ and RS did not significantly affect concentration of volatile fatty acids in ruminal fluid including acetic, propionic, and butyric acids ($P > 0.05$) (Table 3). However, acetic acid concentration in $NaHCO_3$ and $CaCO_3$ groups tended to be lower than those without RS supplementation. Furthermore, there was a tendency for the lowest acetic acid concentration in ruminal fluid of cows fed PP supplemented with $CaCO_3$ (Table 3). This may reflect low buffering ability of $CaCO_3$ because rumen pH is greater than 6.0 (Rogers *et al*., 1985). The ratio of acetic acid (A) to propionic acid (P) was in the range of 2.18 to 2.96 which was not significantly different ($P > 0.05$) (Table 3). The ratio of acetic acid to propionic acid reflects the pattern of ruminal fermentation and ratio of roughage to concentrate in total feed.

Table 3: Influence of treatments on body weight, rumen pH, VFA, blood metabolites and milk of dairy cows

Items	T1	T2	T3	T4	SE	*P-value*
Initial BW, kg	443.00	441.00	449.00	442.00	9.13	0.629
Final BW, kg	446.00	449.00	445.00	456.00	12.10	0.606
BW change, kg.d^{-1}	0.28^{a}	1.28^{b}	-0.26^{a}	1.69^{b}	0.79	0.042
Rumen pH and VFA concentration						
Rumen pH	6.68	6.70	7.12	6.62	0.34	0.225
Acetic, *mmol.l*$^{-1}$	90.64	81.91	76.09	96.05	8.89	0.906
Propionic, *mmol.l*$^{-1}$	35.69	37.60	30.80	32.41	4.36	0.251
Butyric, *mmol.l*$^{-1}$	3.52	2.61	2.72	3.25	2.37	0.868
A:P ratio	2.53	2.18	2.47	2.96	0.57	0.255
Blood metabolites and electrolytes						
Glucose, *mg.dl*$^{-1}$	67.50	65.25	69.50	64.75	3.78	0.348
BUN, *mg.dl*$^{-1}$	7.75	7.25	7.75	8.75	2.64	0.875
Sodium, *meq.l*$^{-1}$	141.25	141.25	139.75	141.75	1.32	0.263
Chloride, *meq.l*$^{-1}$	101.00	96.5	100.25	103.75	5.71	0.421
Bicarbonate, *meq.l*$^{-1}$	23.50	23.75	22.50	23.50	1.75	0.757
Calcium, *mg.dl*$^{-1}$	9.45	9.55	9.40	9.90	0.46	0.473
Potassium, *meq.l*$^{-1}$	4.35	4.22	4.70	4.70	0.24	0.071
Milk yield and composition						
Milk yield, kg	9.18	9.49	9.53	10.00	1.47	0.887
4 % FCM , kg	8.79	9.16	9.16	10.08	1.32	0.591
Fat, %	3.70	3.80	3.73	4.05	0.46	0.711
Protein, %	2.70	2.91	2.95	2.97	0.79	0.622
Lactose, %	4.73	5.14	5.25	5.06	0.44	0.443
Solid not fat, %	8.13	8.75	8.90	8.72	0.65	0.436

T1 = PP to CL ratio of 70:30, T2 = PP to CL ratio of 70:30 with 1.2 % $NaHCO_3$ supplement, T3 =, PP to CL ratio of 52.5: 30 with supplement of 17.5 % RB and 1.2 % $CaCO_3$ mixture, T4 = PP to CL ratio of 50:30 with 20 % RS.
Within rows, means followed by different letters are significantly different at $P < 0.05$.

Russell (1998) reported that the ratio of acetic acid to propionic acid in cows fed 100 % hay was 4.1 while in cows fed 90 % concentrate was 2.2.

Blood metabolites including glucose and blood urea nitrogen (BUN) were unaffected by the treatments ($P > 0.05$) (Table 3). Glucose ranged from 64.75-69.50 mg.dl^{-1} while BUN ranged from 7.25-8.75 mg.dl^{-1}. Kronfeld *et al*. (1982) reported nutritional status of dairy cows indicated by analysis of blood and suggested that normal glucose and BUN should range between 43-69 and 2-22 mg.dl^{-1}, respectively. Electrolytes showed no significant differences among treatments ($P > 0.05$) (Table 3). Serum sodium concentration was unchanged by treatments. In addition, there was no difference in blood chloride, bicarbonate, calcium and potassium among treatments ($P > 0.05$). The normal ranges of serum sodium, chloride, bicarbonate, calcium and potassium are 0.70-157 meq.l^{-1}, 93-152 meq.l^{-1}, 11-26 meq.l^{-1}, 8.5-14.7 mg.dl^{-1} and 1.7-4.5 meq.l^{-1}, respectively (Kronfeld *et al*., 1982).

The supplementation of $NaHCO_3$, $CaCO_3$ and RS did not influence milk yield of dairy cows ($P > 0.05$) (Table 3). However, there was an increasing trend in daily milk production in those cows supplemented with $NaHCO_3$, $CaCO_3$ and RS. Cows fed PP with $NaHCO_3$, $CaCO_3$ and RS produced slightly more milk than cows fed only PP (3.4, 3.8, and 8.9 % or 0.31, 0.35, and 0.82 kg.d^{-1}, respectively). The composition of milk including fat, protein, lactose, solid not fat and total solid was not affected by added $NaHCO_3$, $CaCO_3$ and RS ($P > 0.05$). These data are in agreement with previous reports (Erdman *et al.*, 1982; Rogers *et al*., 1985).

CONCLUSION

Supplementation of $NaHCO_3$, $CaCO_3$ as dietary buffers or RS as saliva secretion stimulant had no significant impact or major physiological changes on performance of lactating dairy cows. No sign of acidosis was observed by added $NaHCO_3$, $CaCO_3$ or RS. However, this experiment was quite limited in experimental animals and time for data collection. Further research should be conducted to test the influence of such diets on milk production with larger number of animals in longer period of time.

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HEAT TOLERANCE TRAITS AND TICK INFESTATION IN SOME INDIGENOUS BREEDS OF CATTLE IN NIGERIA

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ABSTRACT

Ticks are blood-feeding ectoparasitic Arthropods of the subclass *Acari (Arachnida)* that have both domestic and wild animals as hosts. The present study aimed at evaluating the heat tolerance traits and tick infestation in some Nigerian indigenous cattle breeds found in Nasarawa State, north-central Nigeria. A total of 160 animals (83 males and 77 females) comprising 92 Bunaji, 50 Sokoto Gudali and 18 Muturu breeds of Nigerian cattle were sampled. The data were obtained on the number of tick counts on each animal, as well as physiological parameters, such as rectal temperature, respiratory rate and pulse rate. This study revealed the presence of two cattle tick species *Dermacentor andersoni* (58, 53.2 %) and *Ornithodorus moubata* (51, 46.8 %), which are of veterinary importance. The prevalence was highest in the Bunaji breed (71, 65.1 %), followed by Sokoto Gudali (26, 23.9 %) and Muturu (12, 11.1 %) cattle, respectively. Occurrence of tick infestation was higher in male (59, 71.1 %) than female animals (50, 64.9 %). Respiratory rate and pulse rate were significantly ($P < 0.05$) higher in Muturu cattle compared to the Bunaji and Sokoto Gudali cattle. Sex effect on the physiological parameters was not significant. Animals infested with ticks had significantly higher rectal temperature (39.01 ± 0.14 versus 38.40 ± 0.12 °C). Rectal temperature and breed were found to be more associated with the incidence of tick infestation in the binary logistic regression. The present findings may aid the design of effective control measures against ticks and subsequent breeding for genetic resistance to tick infestation.

Key words: cattle; Nigeria; physiological traits; prevalence; ticks

INTRODUCTION

Cattle are the most important species of ruminants in Nigeria (Yakubu *et al.*, 2010). Infestations by ectoparasites are among the main problems that affect stock raising in tropical countries (Jonsson, 2006; Bianchin *et a*l., 2007). There are almost 900 species of ticks that are endemic to most continents (Barker and Murrell, 2004) and nearly every country has at least one species of tick among its fauna (Krcmar *et al*., 2014). Ticks of the family *Ixodidae* (hardbodied) have a one-, two- or three-host life cycle (Minjauw and McLeod, 2003), while ticks of the family *Argasidae* (softbodied) are "free living" but remain in close proximity to their host, e.g. at their host nest (Jongejan and Uilenberg, 2004). The pathogenic effects of tick infestation are associated with the feeding pattern of the parasite, which is ideal for both penetrating the skin and transmitting microorganisms (Lysyk, 2013).

A large component of the economic cost of ticks in cattle is the application of control measures to reduce infestations (De Castro, 1997) and the basis of most is the application of chemical acaricides. The emergence of acaricide-resistant strains of ticks, increased scrutiny of chemical residues in livestock products, and increased cost of acaricides, has driven the search for alternate strategies to control ticks. The concept of integrated tick control has been widely promoted, involving combinations of chemicals, environmental management, vaccines against tick antigens, biological

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control measures and the utilization of cattle genotypes with increased resistance to ticks (Jonsson and Piper, 2007). Tick resistance among cattle is influenced by a number of factors. The most important are increased levels of histamine at the early stages of the infestation, self-cleaning behaviour, increased levels of eosinophils, basophils and mast cells, the presence of specific immunoglobulin patterns, T cells and genes related to the expression of keratins and lipocalins (Kashino *et al*., 2005; Piper *et al*., 2010; Kongsuwan *et al*., 2010).

In Nasarawa State, north-central Nigeria, there is dearth of information on tick infestation in cattle. Better understanding of prevalence of tick infestation will help in efforts to control the parasite. The study aimed at investigating the prevalence of tick species in Lafia, Nasarawa State and examining the effect of breed, sex and tick infestation on some physiological parameters, such as rectal temperature, respiratory rate and pulse rate. The association of breed, sex, rectal temperature, respiratory rate and pulse rate on tick infestation in the study area was evaluated.

MATERIAL AND METHODS

Study area

The study was conducted between May, 2013 and March, 2014 at the Cattle Unit, Livestock Complex of the Department of Animal Science, College of Agriculture, Lafia; Cattle market, Lafia and Namaledu Cattle Fattening Center, old Alhamis Market, Lafia, Nasarawa State, Nigeria. Lafia has a tropical climate. It is located within latitude 8° 29‘ 24“ N) and longitude (8° 31‘ 12“ E) at an altitude of 181.53 m (570 ft) above the sea level. The major climatic elements of the area include rainfall, temperature, wind speed and relative humidity. The mean annual rainfall ranges between 1270 and 1530 mm (Ezeaku and Salau, 2005). The dry season lasts from November to March and is characterized by the North-East trade wind, which brings harmattan in November and December. The wet season begins in April and ends in November and is characterized by South-West wind. The temperature of the area varies from 23.7 °C to 31.1 °C. The area showed low temperature in June to September that rises in November to April. The relative humidity of the area ranges from 27 % to 89 %; wet season showed the range of 71-89 %, while the dry season showed range 27-71 %. The relative humidity is high during the wet season. Wind speed varies between 39.12 $m.s^{-1}$ and 93.8 $m.s^{-1}$ for both dry and wet seasons. The daily mean duration of sunshine is about 11.5 hours.

Experimental animals

A total of 160 animals (83 males and 77 females) comprising 92 Bunaji, 50 Sokoto Gudali and 18 Muturu breeds of Nigerian cattle were randomly sampled. All the animals sampled were within the 8-tooth age (greater than 48 months old). The age of the animals was determined using permanent dentition. They were allowed to graze natural pasture while this was supplemented occasionally with local concentrates, such as dusa (fermented sorghum waste) and groundnut haulms. There was also occasional supply of drinking water. The animals were not subjected to any acaricide treatment during the period of the experiment. Apart from physical and physiological observations, blood samples were not collected from the animals to detect their health status.

Physiological parameters

Physiological parameters (rectal temperature, respiratory rate and pulse rate) were taken on all the animals sampled. Rectal temperature was measured using a digital thermometer. The sensory tip was disinfected and inserted into the rectum at the display of L°C by a thermometer (when the digital thermometer is activated, a beep will sound and the display will be in the ready mode as indicated by a “L” symbol showing that the thermometer is set for temperature reading). This was removed after the sound of the alarm signal. The displayed body temperature was then recorded. Respiratory rate was determined by counting the number of flank movements per minute. Pulse rate was measured by placing the fingertips on the femoral arteries of the hind limb for 1 min. The physiological parameters were taken as earlier described (Olson *et al*., 2002).

Ticks identification

Tick samples were also collected from each animal and put into a sample bottle containing 70 % alcohol. The sampling bottles were properly labelled indicating the area of collection, type of parasite and the date of collection. The ticks were then taken to the Zoology Unit of the Faculty of Biological Sciences, Nasarawa State University, Keffi for Laboratory identification. The ticks were taxonomically identified, as described by Medler (1980). Anderson´s (2004) method of expressing prevalence and intensity was adopted.

Statistical analysis

Data obtained were analyzed by simple averages and percentages according to breed, sex of cattle and tick species. The analysis of variance (ANOVA) was used to assess the effect of breed, sex and tick infestation on rectal temperature, respiratory rate and pulse rate.

The general model employed was:

$Y_{ijk} = \mu + B_i + S_j + T_k + e_{ijk}$

Y_{ijk} = individual observation

μ = overall mean

B_i = fixed effect of ith breed (i = Bunaji, Sokoto Gudali, Muturu)

S_j = fixed effect of jth sex (j = male, female)

T_k = fixed effect of kth tick infestation

e_{ijk} = random error associated with each record (normally, independently and identically distributed with zero mean and constant variance).

The logit of the probability of tick infestation was modelled using logistic regression assuming an asymptotic binomial distribution. First, the univariate analysis for all hypothesized risk factors (breed, sex, rectal temperature, respiratory rate and pulse rate) and the occurrence of ticks in the present study was carried out using Pearson's Chi-square (χ^2) test. Subsequently, a multivariate model was built by including every hypothesized risk factor which had p-value of $P < 0.20$ from the univariate analysis. Backward stepwise elimination based on Wald method was applied (Noordhuizen *et al*., 2001). The Chi-square goodness-of-fit test was performed to check if the multivariate logistic model fit the data well ($P > 0.05$) (Hosmer and Lemeshow, 2000). The multivariate model (Czopowicz *et al*., 2012) employed was:

$$P(Y=1) = \frac{1}{1 + \exp[-(B_0 + B_1 \times X_1 + ... + B_n \times X_n)]}$$

where,

P (Y=1) = probability of a final outcome (Tick infestation)

B_0 = intercept

B_1, B_n = regression coefficients for individual risk factors

X_1, X_n = risk factors (breed, sex, rectal temperature, respiratory rate, pulse rate)

The statistical package employed in the analysis was SPSS (2010).

RESULTS

The prevalence of ticks according to the breed of cattle examined in Lafia, Nasarawa State, Nigeria indicated that of 160 cattle infested, the prevalence was highest in the Bunaji breed (71, 65.1 %), followed by Sokoto Gudali (26, 23.9 %) and Muturu (12, 11.1 %) (Table 1).

The prevalence of ticks according to the sex of the animals examined in the two states indicated that of 83 males examined, 59 (71.1 %) were infested, whilst of 77 females examined, 50 (64.9 %) were infested (Table 2). Two major tick species were taxonomically identified and confirmed as *Dermacentor andersoni* (58, 53.2 %) and *Ornithodorus moubata* (51, 46.8 %), respectively (Table 3).

Table 1: Prevalence of tick infestation according to breeds of cattle examined in Lafia, Nasarawa State

Breed	No. (%) of male infested	No. (%) of female infested	Total (%) infested
Bunaji	32 (54.2)	39 (78.0)	71 (65.1)
SokotoGudali	19 (32.2)	7 (14.0)	26 (23.9)
Muturu	8 (13.6)	4 (8.0)	12 (11.1)
Total	59 (54.1)	50 (45.9)	109

Table 2: Prevalence of tick infestation according to sex of cattle examined in Lafia, Nasarawa State

Sex	Total number sampled	No. (%) of infested
Male	83	59 (71.1)
Female	77	50 (64.9)

Table 3: Occurrence of tick species among cattle in Lafia, Nasarawa State

S/N	Species	No. of Animals infested	No. Male infested	No. female infested
1	*D. andersoni*	58 (53.2)	26 (44.8)	32 (55.2)
2	*O. moubata*	51 (46.8)	28 (54.1)	23 (45.1)

Table 4: Effect of breed, sex and tick infestation on the physiological parameters of Nigerian indigenous cattle

Parameters	Rectal temperature (°C)	Respiratory rate (breaths/minutes)	Pulse rate (beats/minutes)
Breed			
Bunaji	38.58 ± 0.12^{a}	15.75 ± 0.21^{b}	48.95 ± 0.44^{b}
SokotoGudali	39.07 ± 0.21^{a}	16.20 ± 0.27^{b}	49.16 ± 0.62^{b}
Muturu	39.04 ± 0.31^{a}	19.06 ± 0.74^{a}	54.50 ± 1.25^{a}
Sex			
Male	38.67 ± 0.15^{a}	16.36 ± 0.25^{a}	49.31 ± 0.53^{a}
Female	38.97 ± 0.15^{a}	16.16 ± 0.28^{a}	49.99 ± 0.53^{a}
Tick infestation			
Tick –	38.40 ± 0.12^{b}	16.10 ± 0.33^{a}	49.94 ± 0.63^{a}
Tick +	39.01 ± 0.14^{a}	16.34 ± 0.23^{a}	49.50 ± 0.46^{a}

– = absence of ticks, + = presence of ticks
[ab]Means along the column with the different superscripts are significantly ($P < 0.05$) different

Table 5: The association between variables and the prevalence of tick infestation in Nigerian indigenous cattle*

Parameters	Pearson's Chi-square	P-value
Breed	10.44	0.01
Sex	0.44	0.51
Rectal temperature	32.79	0.09
Respiratory rate	14.02	0.37
Pulse rate	22.25	0.57

*Only parameters with $P < 0.2$ were included in the subsequent multivariate logistic regression analysis.

Effects of breed, sex and tick infestation on the physiological parameters of Nigerian indigenous cattle are shown in Table 4. Respiratory rate and pulse rate were significantly ($P < 0.05$) higher in Muturu cattle compared to the Bunaji and Sokoto Gudali cattle. Sex effect on the physiological parameters was not significant ($P > 0.05$). Animals infested with ticks had significantly ($P < 0.05$) higher rectal temperature. Tick infestation, however, did not affect significantly ($P > 0.05$) respiratory rate and pulse rate.

Following univariate statistical analysis, breed and rectal temperature were the eventual parameters fitted into the multivariate logistic regression models based on the significance level $P < 0.20$ (Table 5). The logistic regression models showed that rectal temperature (odds ratio = 2.68; P = 0.00) and breed (odds ratio = 0.35; P = 0.00) were associated with the prevalence of tick infestation (Table 6). The model appeared reliable, as revealed by the Hosmer and Lemeshow´ test: $\chi^2 = 5.12$, P = 0.74.

Table 6: Logistic regression predicting the prevalence of tick infestation in Nigerian cattle

Parameters	B	S.E.	Wald' χ^2	P-value	Odds ratio	CI (95 %)
Intercept	-35.53	9.99	12.65	0.00	0.00	
RT	0.98	0.27	13.79	0.00	2.68	1.59-4.50
Breed	-1.04	0.30	12.39	0.00	0.35	0.20-0.63

RT = rectal temperature, B = regression coefficient, S.E. = standard error of B, CI = confidence interval
Hosmer and Lemeshow test: $\chi^2 = 5.12$, P = 0.74

DISCUSSION

This is the first report of *Dermacentor andersoni* and *Ornithodorus moubata* in Nasarawa State, Nigeria. Ticks are among the most important ectoparasites and vectors of animal and human diseases on global scale, particularly in tropical and sub-tropical parts of the world. Because of the direct and indirect effects on their hosts, they are considered to be a significant threat to successful livestock production and seriously interfere with the economy of a country (Feleke, 2003). In Nigeria, 90 % of the cattle population is kept under the traditional pastoral husbandry of Fulani herders. Under the Fulanis' management, cattle are extensively grazed in pastures and forest, and exposed to infestation by ticks (Lorusso *et al.*, 2013). The distribution of ticks among Bunaji, Sokoto Gudali and Muturu suggested that tick infestation was more common in Bunaji than other breeds. It seems that the white color of Bunaji breed of cattle plays a vital role in attracting more ticks to it. White objects reflect light, making the body of the object to be cooler than black or other colored objects, which seem to absorb light, thus conserving heat. Therefore, breeding for genetic resistance against tick infestation is imperative. According to Ibelli *et al.* (2011), the use of tick resistant genetic groups could be an alternative to increase the productivity of cattle in crossbreeding systems without increasing the use of acaricides.

The present results agreed with the data of Hitcheock (1993), who reported that male cattle are more infested with ticks than the females, because most of the males in the tropics are always moved from place to place in search of food and in this process get infested with ticks, while the females are confined mainly for breeding purposes. *Dermacentor andersoni* and *Ornithodorus moubata* were the two tick species found in this study. The distribution of ticks within a specific habitat depends on several environmental and climatic factors, such as annual rainfall, atmospheric temperature and relative humidity (RH), vegetation cover, altitude and host availability (Lorusso *et al.*, 2013; Iqbal *et al.*, 2014).

The higher respiratory rate and pulse rate of Muturu indicate that they were more stressed than the two other breeds. Animals function most efficiently within their thermoneutral zone, while above the upper and the lower critical temperatures are stressful for animals, and therefore the environment constrains the production process. However, those critical temperatures are not fixed characteristics for any species or animal type and they may change with age and physiological conditions. Natural and artificial selection in an extreme environment can improve adaptation for those conditions in terms of adaptive morphological and physiological traits of livestock (Silva *et al.*, 2007).

The correlation between the tick infestation and genetic group was positive and significant (Veríssimo *et al.*, 2002). Rectal temperature and breed have been found to be important in predicting tick infestation in the present study. The higher the rectal temperature, the more disposed an animal is to tick infestation. Bunaji cattle were more disposed to tick infestation compared to the two other breeds. The prospects for increased productivity based on efficient and sustainable exploitation of cattle inherent unique features, such as adaptability, ability to thrive in harsh environmental conditions, resistance to parasites and disease etc. should have the objective of increasing cattle population.

CONCLUSION

This study ascertained the presence of two cattle tick species, which are of veterinary importance. The prevalence of tick infestation was highest in the Bunaji breed than the Sokoto Gudali and Muturu cattle breeds. Prevalence of tick infestation was higher in male than female animals. Respiratory rate and pulse rate were significantly higher in Muturu cattle compared to the Bunaji and Sokoto Gudali cattle. Sex effect on the physiological parameters was not significant.

Animals infested with ticks had significantly higher rectal temperature. Rectal temperature and breed were found to be more associated with the incidence of tick infestation in the multivariate logistic regression. It is important for the relevant authorities in Nasarawa State, north-central Nigeria to design effective control measures against these ticks, with full knowledge of their biology. Breeding for genetic resistance to tick infestation should also be considered in the long run.

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ADAPTABILITY OF DAIRY COWS TO ROBOTIC MILKING: A REVIEW

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ABSTRACT

Robotic milking systems (RMS) offer an innovative approach to improve productivity on dairy farms. RMS will influence the future growth of farms, the nature of husbandry employed and the quality of life on family farms. For farms using hired labour and located near industrial centres, RMS may contribute to the reduction of wage costs. As this technology is very expensive, and little is known regarding its interplay between animal, technology and stockman, or the effects on milking performance the detailed study is very required. Behavioural observation of the animals and monitoring of the system become extremely useful. In this review, the RMS impact on dairy cows is explored. What is the basis for adapting to the dairy cow milking is discussed in the first part of this review. Some housing parameters related to structures, design and environment are reviewed. Behavioural requirements of cows for robotic milking are written in the second part. The third part highlights the anticipated problems and last part is devoted to adaptability of cows to robotic milking. Recent studies on the impact of automated milking, different management regimes, and relocation with milking manner change on behaviour of dairy cows are discussed. The effects of inadequate milking procedures and improper milking technical parameters on welfare and udder health of cows are also emphasized.

Key words: dairy cow; robotic milking; housing; behaviour

INTRODUCTION

Husbandry intensification is often believed to lead to a reduction in animal welfare. We understand that every type of housing system must provide conditions conducive to comfort, good health, growth and performance at all stages of the animal‘s life. Automatic milking systems or robotic milking systems (RMS) offer an innovative approach to improve productivity on dairy farms. As this technology is very expensive, and little is known regarding its interplay between animal, technology and man, or the effects on milking performance, the detailed study is very important. Behavioural observation of the animals and monitoring of the system become extremely useful.

There is no doubt that RMS has become an important practice in dairy production. An increase of desire for a social life and more freedom has led many farms to take advantage of a new technology. Two-thirds of farmers think that a better social life is their reason for investing in an RMS (Mathijs, 2004). A single stall robot system can milk 55-65 cows per day, so this may be one of the reasons why adaptation is most prevalent in Northern and Western Europe as the farms are at suitable size.

In 1992, the first commercial milking robots came into use. Since 2000, RMS have substantially increased in popularity, and as in 2012, there were more than 10 000 farms in 25 different countries using RMS. Swedish and Dutch factories (Alfa Laval and Lely)

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had sold more than 19 500 robots by the end of 2012. In the Czech Republic 170 robots have been working. However, the Slovak Republic lags far. At present there are only 16 robots.

Robotic milking is a suitable solution also for large dairies, as there are several examples of successful dairies working under different conditions around the world. While technology costs go down, labor costs go up. This irreversible trend has affected most industries, and dairy is no exception. Automation will be the sustainable solution of the future. In addition to lower labour requirements, RMS results in a 5-10 % increase in milk yield which is mainly due to the increased milking frequency (Ketelaar de Lauwere *et al*., 2000; de Koning, 2010).

Milking, milk yield, milk quality, cow traffic, behaviour, and coping on environment are essential elements of RMS. When evaluating the performance tests, we focus not only on milk yield and milk quality, but also to changes in live weight of dairy cows and of course on health. There is limited information of behavioural factors associated with change of milking system after relocation of lactating dairy cows. Therefore, we focused on a detailed assessment of the adaptation of dairy cows to RMS.

The basic assumptions for use

Undoubtedly, automatic milking changes many aspects of farm management since both the nature and organization of labour is altered. Manual labour is partly replaced by management and control, and the presence of the operator at regular milking times is no longer required. Cow management including routing within the barn, the opportunity for grazing and the use of total mixed rations is altered. A high level of management and realistic expectations are essential for successful adoption of automatic milking.

When the cow enters the milking station (encouraged by highly palatable feed in the milking station), an ID sensor reads the cow's identification tag (transponder). These data go to the computer. If the cow was milked recently, the automatic gate system would send it out of the unit without access to feed or having been milked (Mihina *et al.*, 2012). When a cow milking is allowed and cow walks in the RMS to be milked, a 3D camera and laser technology helps the robotic arm to track the cow's movements and locate each teat. It attaches the teatcups and then starts milking each quarter at a time, adjusting the pulsation ratings for each quarter. This results in both optimal milking and more gentle experience for the cow (André *et al.*, 2010). Facilities for teat cleaning and separation of abnormal milk are incorporated into the automatic system and several adaptations are needed to accommodate continuous milking. RMS-systems are also equipped with sensors to observe and to control the milking process. Data are automatically stored in a database and the farmer has a management program to control the settings and conditions for cows to be milked. Attention lists and reports are presented to the farmer by screen or printer messages.

Visual control of cow and udder health at milking is, at least partly, taken over by automatic systems. The farmer‘s presence at regular milking times is no longer required. The nature and organization of farm labour changes such that manual labour dealing with milking is largely replaced by management and control activities. Regular visual checks of cow and udder health during milking are taken over by automated monitoring using smart sensor technology.

Well-functioning cow traffic is a prerequisite for a successful RMS. This includes an optimal number of visits both to the feeding area (number of meals) and to the RMS for all cows in the herd when cows are kept indoors, as well as during grazing. Cows housed in a free stall barn with voluntary visits to feeding and milking areas develop individual patterns of eating and diurnal activity over time (Melin *et al.*, 2005).

After visiting the milking system, the cow should have access to the feeding area. In "forced traffic" systems she has to pass the milking system in order to get access. In "controlled traffic" systems one-way-gates, with cow identification and selection capabilities, restrict cows to go directly to the feeding area only when the interval since the last milking exceeds the pre-set minimum.

Behavioural requirements of cows for robotic milking

Cow needs to have good locomotion (Tongel and Broucek, 2010; Micinski *et al*., 2010; DeVries *et al*., 2012). Voluntary cow movement has a strong influence on robot utilization (Holloway *et al*., 2014). The introduction of RMS brings about a significant alteration in the way cows are milked; no longer are they driven to a parlour by the farmer, but they walk to the RMS on their own accord. Moving of cows, which increases labour requirement, should be minimalized. Cows must make their own way to the milking unit and stand quietly while being milked. This requires emphasis on traits, especially temperament (Adamczyk *et al*., 2013; Broucek *et al*., 2008).

A behavioural indicator of particular relevance to robotic milking systems is the time budget of the cows, i.e. how much time they spend in different basic activities. Increased standing time may indicate stress or discomfort, and cows of low social rank spend more time standing, because they have to wait in front of the robot. Also, it is extremely important to determine how behaviour is influenced by numerous milking. The lying behaviour patterns of cow milked

in a RMS would be different from those milked in a conventional system, particularly since the distribution of milking events in an RMS occurs over a 24 h period. DeVries *et al*. (2010) noted less synchrony in the behaviour of RMS milked cows, resulting in less daily time spent lying down. Also, some cows tend to be less active after midnight, so robots are often idle during the early hours of the morning.

Usage of an RMS requires cows to be more self-motivated and independent in contrast to cows being milked in a conventional parlour. Therefore, their behaviour and temperament especially is one of the most substantial concerns in the increasing popularity of the RMS. Albright and Arave (1997) describe temperament as a set of behavioural characteristics that contribute to the unique disposition of one animal in contrast to other species members. Another has identified the key parts of temperament as being docility, workability, disposition and fearfulness (Ketelaar-de Lauwere *et al*., 1996; Rushen *et al*., 1999). Cow's temperament consists of multiple traits: fearfulness, activity, sociality (Scott *et al.*, 2014).

Appropriate social behaviour is especially important in a RMS because competition at the entrance occurs. In a study by Bach *et al.* (2009), cows with a higher dominance value spent less time in the waiting area. Ketelaar-de Lauwere (2000) demonstrated that the type of RMS has a marked influence on the cows' subsequent behaviour. Non-milking visits and failed attachments were followed more often by incomplete behavioural cycles. Stefanowska *et al*. (1999a) found that a missed milking negatively influenced cow behaviour, such as less time spent lying and more frequent urinating. Longer post-milking standing durations were associated with cows of higher parity (Norring *et al*., 2012; Deming, 2013).

Social order plays a strong role in determining an individual animal's access to a resource, and in competitive situations (Broucek *et al*., 2011). Low-ranked cows spent more time in the waiting area, while high-ranked cows spent more time in the resting area (Prescott *et al*., 1998; Rushen *et al*., 1999b). The physical restrictions given by the size, use and the design of different parts of the barn have a major impact on the social behaviour of housed cows (Manteca and Deag, 1993). Installing robots not only changes the way the operation runs, but more importantly, it allows each cow to reveal her natural behaviour (DeVries *et al*., 2011).

Some studies found higher levels of restlessness behaviour such as stepping, foot-lifting and kicking in RMS than in a milking parlour. Stepping during milking can be used as an indicator of general discomfort and fear towards humans. A higher frequency of stepping behaviour is observed in anxious and nervous animals. Cows which are managed gently show a shorter flight distance and less stepping behaviour during milking (Phillips and Rind, 2001). Note that cows which experience pain due to teat lesions are more likely to kick during milking. Kicking is also an indicator of discomfort caused by low milk flow and vacuum milking. Defecation, urination and vocalization in RMS are parameters of acute stress and fear in cows. These measures increase when the cows are isolated or introduced in novel surroundings (Munksgaard and Jensen, 1996; Kilgour, 1998; Broucek *et al*., 2003).

Kicking of the cow while inside the RMS can present many problems. Kicking can cause damage to both the teat cleaning devices and the teat cups. It can result in incomplete milking and consequently less milk yield, as well as longer attachment time (Watters *et al*., 2013). Not just kicking in the RMS could indicate that cows are uneasy while in the milking robot. In a study by Wenzel *et al*. (2003), it was shown that cows using milking robots stepped more in the milking robot than in a conventional parlour.

Robotic milking machines are novel technologies that take over the labour of dairy farming and reduce the need for human-animal interactions. The introduction to a new housing system is being exposed to new human handlers. Cattle show individual variation in their behavioural responses to handling and management systems on farms. These behavioural responses are presumed to reflect underlying temperament traits such as fear or aggression. Handling problems cause higher labour costs, injuries to stockpersons and cattle or even deadly accidents. Therefore, farmers are demanding docile cattle with "good temperament", which enable easy, safe and fast handling (Wechsler and Lea, 2007). Dairy cows were found to keep a longer distance from an aversive than a gentle handler (Munksgaard *et al*., 2005; Rushen *et al*., 2012).

The presence of an aversive handler (sudden and unpredictable movements, shouting and/or slapping) during milking is sufficient to cause the cows to "hold-back" milk due to the suppression of oxytocin secretion. Studies comparing farms with similar environmental conditions and cows with the same genetic background have shown that farms with the highest production are those with stockpersons that speak to and touch their cows more often. The animals are, in turn, less frightened, less reluctant to being driven and more likely to approach the stockperson. Under controlled conditions, just the presence of an aversive handler during milking is sufficient to increase residual milk by 70 % and therefore reduce milk yield (Munksgaard *et al*., 2001; Wechsler and Lea, 2007; Rushen *et al*., 2012).

The behavioural response of cows to a stressful situation increases the risk of injury for the stockman. The expression of fearfulness is the result of interactive processes related to past experiences and the animal's genetic background (Hemsworth *et al*., 1995; Burrow, 1997; Broucek *et al.*, 2004). Cattle show individual variation in their behavioural responses to handling and management systems on farms. However, dairy cows kept in large herds, and especially primiparous cows are more prone to exhibiting behaviour that can compromise both stock person's safety and animal welfare (Sabbioni *et al.*, 2012; Popescu *et al*., 2013).

What to pay attention on

From a technical point of view RMS is proposed perfectly, but requires improvement in terms of the protection and welfare of dairy cows. Cows milked more than twice per day produced more milk (Hart, 2013). However, several cows are milked less than twice a day in RMS and may therefore produce less milk. Robotic herding would improve animal well-being by allowing cows to move into RMS whenever. However, the teats could be sore from mastitis or of being milked too frequently. If the robot can't attach optimally milking speed goes down, stay time in RMS increases and if the cows are not milked properly they might develop mastitis which can be resulted in milk quality problems.

Milking speed is also affected by the cow's temperament; a nervous animal will have an increased level of adrenalin, which can block the oxytocin reflex and interrupt the milk let-down (Falkenberg *et al*., 2013). There is a risk for failure of milking, such as missed attachment of the milking cluster.

Although the benefits reaped from a RMS are extensive, there are a few drawbacks. Milk quality is a critical concern on modern dairy farms, because milk payment systems are based on milk quality and consumers expect a high level of quality and safety from the milk products. Milk quality can be reduced in some cases, welfare and health can be compromised. Although RMS uses the same milking principles as conventional milking, there are major differences. The RMS is in use for 24 hours continuously. Visual control during the milking process is not possible. Cows will visit the RMS more or less voluntarily and this will result in a big variation in the milking frequency from cow to cow. All these aspects may influence the quality of the milk produced (Klungel *et al*., 2000; De Koning, 2010).

A variety of stressors, such social isolation, novel surroundings (especially for heifers) or fear of people present at milking lead to an inhibition of milk ejection. Chronic pain associated with diseases or injuries and any stressful situations occurring during milking are likely to produce a decrease in milk yield. Acute stress during milking reduces milk yield through a central inhibition of oxytocin secretion and peripheral actions of catecholamines. Oxytocin, which is a hormone secreted by the central nervous system into the blood stream, is the main mediator of the milk ejection reflex. The secretion of oxytocin is then of major importance to optimize milk production (Falkenberg *et al*., 2013). Changes in the milking parlour can also affect cow behaviour after relocation (Hillerton *et al*., 2001). Being milked in an unfamiliar environment can cause the inhibition of milk ejection (Macuhova *et al*., 2008).

A lame cow is unwilling to move and will not go to the robot. Udder conformation has improved a lot with modern genetics, but there has been a selection for bulls and cows that transmit close rear teats, which works well in a parlour, but can be difficult to find for the robot (Bijl *et al*., 2007; Weary *et al*., 2009; Tongel and Broucek, 2010). Teat size and adequate teat placement is also important, and good consistency from cow to cow is preferable; if teat sizes vary a lot it can be difficult to choose the right liner (Mihina *et al*., 2012; Caria *et al*., 2014).

With respect to the welfare of the dairy cow, the use of RMS has both advantages and disadvantages. Some recent studies conclude that automatic milking and conventional milking are equally acceptable in terms of welfare of the dairy cow (Pastell *et al*., 2006; Jago and Kerrisk, 2011). Stressed or uncomfortable cow might start kicking in the RMS machine. It prolongs the milking time and number of milkings goes down. Long waiting in the holding area and in the RMS after milking caused stress in animals. This will impact the milk yield, resulting in less efficiency, a decrease in production and lower profitability. Cows may be unwilling to enter a milking parlour voluntarily after negative experiences with inconvenient human contact (Broucek *et al.*, 2008).

According to our results (Broucek *et al*., 2013a), cows of high social rank entered the milking parlour more often without spending time in a queue. In contrary, cows of low social rank had a longer total daily waiting time in front of the RMS. Also, these submissive cows could spend less time in the resting area. The overcrowding develops psychological stress in animals. Increasing of animal density after milking manner change can lead to a decrease in the amount of lying time per day. Every movement of animals and their grouping makes confusion among them. For example, some authors (Bouissou *et al*., 2001; Huzzey *et al*., 2006; Neisen *et al*., 2009) showed that high stocking density at the feeding alley influenced negatively the feeding time and the competition among the cows. Cows ranked lower in the social hierarchy were more often displaced (Galindo and Broom, 2000; Gonzales *et al*., 2003;

DeVries *et al*., 2004). Group housing management and amount of bedding in particular, can have a significant effect on the comfort of cows, as well as free-stall associated lying behaviour (Ketelaar-de Lauwere *et al.*, 1996; Fregonesi and Leaver, 2002; Lendelová and Pogran, 2003; von Keyserlingk and Weary, 2009). Potter and Broom (1987) and Phillips and Rind (2001) highlighted the importance of sufficient space for feeding and resting to allow the herd to adaptation.

The RMS required the distribution of cow behavioural activity throughout the day. The maintenance behaviour patterns of cows could be changed (Adamczyk *et al.*, 2011; Broucek *et al*., 2013b). The synchrony behaviour could be different. Cows do not disturb each other. This would impair their well-being.

Adaptability of cows to robotic milking

The procedures proposed solutions are highly topical. Many dairy buildings are relatively old and cow freedom is restricted. Therefore, a number of farms will currently change the manner of milking on RMS. However, the relocation process has been implicated as one of the major aversions for received cattle (Grandin, 1999; Broucek *et al*., 2013b). The stress associated with removing and arrival at a new facility with milking change can be one of the most stressful situations an animal experiences and can cause a number of physiological and behavioural changes including altered hormones, parameters of energy and protein metabolism, and also changes in milk production (Koolhaas *et al.*, 2010). During relocation cattle are subjected to noise, strange surroundings, odours and companions, overcrowding or sometimes isolation, hot or cold conditions and a change of feed (Grandin, 2003). All these factors contribute to stress and potential performance losses. Replacing conventional twice-a-day milking managed by people with a system that supposedly allows cows the freedom to be milked automatically whenever they choose, it is claimed that robotic milking has health and welfare benefits for cows, increases productivity, and has lifestyle advantages for dairy farmers (Holloway, 2013).

Cows have two motivations to enter the robot: access to concentrate and emptying of the udder. In standard conditions, concentrate is provided in predetermined quantities to the cow while she is being milked. When feed was provided, cows were faster to exit the pre-milking yard, they have a shorter time spent waiting (Scott *et al*., 2014). In controlled-traffic systems, cows achieve access to the feeding area after being milked. Therefore, this system depends on cows' motivation to eat at regular intervals. With free access, cows do not have to be milked in order to enter the feeding area. This system allows unlimited access to forage. Cows can join the milking robot every time and therefore the interval between milkings is not fixed. The frequency, with which cows choose to be milked, has been reported to range from 1.2 to 5 times a day (Pastell *et al*., 2006).

The cow's motivation to enter the milking stall is the major difference between RMS and conventional milking systems. In conventional milking routines, the cows are driven to the milking parlour two to three times daily. In RMS, the cows enter the milking stall voluntarily and are milked throughout the day without human intervention. It has been demonstrated that cattle can be trained to approach a feed source after hearing an audio signal (Arnold *et al*., 2008). However, some cows have the obvious motivations to visit the robot but are not doing so, especially those who are at peak lactation (Nixon *et al*., 2009). This could indicate that something else is hindering them from entering the RMS. More than 15 % of the herd needs to be forcing to visit. Therefore, training cows to use a RMS is an important process, as this method of milking depends on cows voluntarily using the RMS. Jago and Kerrisk (2011) found that voluntary milkings were achieved by 92 % of heifers and 81 % of cows within 6 days following their first assisted milking. Heifers achieved their first voluntary milking quicker than cows. Pre-calving training improved aspects of the behaviour required for successful adaptation to RMS but had little impact on time to achieve a voluntary milking. Generally, cows must learn to manipulate automatic feeders, water bowls and to enter the milking unit. Therefore, cattle can be trained to perform different kinds of tasks (Kilgour *et al*., 1991; Veissier *et al*., 1993). Operant conditioning techniques have been used on cattle to measure feed preferences (Arave, 1996), handling preferences (Pajor *et al*., 2000) and behavioural needs (Broucek *et al*., 2002; Broucek *et al*., 2003; Wredle *et al*., 2006).

To ensure motivation and performance it is important to develop a consistent training routine for heifers and cows. Training cows to use a RMS is an important process as this method of milking depends on cows voluntarily using the RMS. According to our recent results, we assume that primiparous cows adapted to automatic milking quicker than older cows (Broucek *et al*., 2013a).

We assume that experienced cows will enter the RMS voluntarily without any intervention by the staff or their adaptation period after a transient period of parlour milking will be short. However, main attention should be focused on the behaviour of inexperienced cows. Our preliminary results are alarming, inexperienced cows need an intensive help for adaptation to the RMS in order to minimize production loss. So, the adequate adaptation is crucial for successful milk production in RMS. Time efficiency and time usage in RMS

are key aspects in robotic milking, and a stressed or uncomfortable cow might start kicking in the machine, which will result in longer attachment time.

Heart rate variability is an alternative measure that has been used recently for the evaluation of stress responses in dairy cows (Lay *et al*., 1992; Kovács *et al*., 2013). Weiss *et al*. (2004) found that most cows adapted to the RMS within few days, but there was wide individual variation of heart rate among cows. The adaptability seemed to be related to the individual sensitivity of their adrenal cortex (Hagen *et al*., 2005; Gygax *et al*., 2007).

Cows' performance in housing systems with RMS can depend on their temperamental traits. Avoidance of the RMS can be related to a fear but also to unfavourable temperament. Cows in RMS need to have the same functional traits as cows in other milking systems, but in addition, they also need an appropriate temperament; calm but driven. Environmental conditions which elicit physiological coping responses in animals, it causes deterioration of well-being and slow adaptation of cattle (Albright and Arave, 1997). The first stage is acclimatization to an RMS, which takes a few or more days in most circumstances. However, adapting to a new system may be more difficult for some cows than others (Wenzel *et al*., 2003; Deming *et al*., 2013).

It is necessary to develop methods and procedures for easier adaptation of cows to RMS. More authors reported how habituation was used to encourage dairy cows to enter the automatic milking system (Hagen *et al*., 2005; Scott *et al*., 2014). The problem may be the noise of the robot. Arnold et al. (2008) examined the effect of noise on choice behaviour of dairy heifers in a maze. Animals took longer to enter the noise maze arm compared to the quiet arm during training trials. From studies in calves and heifers in the maze learning it is known that differences in response between animals may originate from rearing conditions (Broucek *et al*., 2002). Broucek *et al.* (2007) indicated that heifers are reluctant to change their initial choice. It is possible that the willingness or reluctance also can be seen in cows. Reaction may be due to previous experience in some food, which was previously received as a reward, but it was not used.

Wicks *et al.* (2004) investigated the effect of habituating heifers to the milking parlour environment prior to calving on subsequent lactation performance. Habituated heifers yielded at 1.3 kg per day more milk than the control group of heifers over the first 100 days of lactation. Results of Sutherland and Huddart (2012) suggest that trained heifers may have experienced less distress during the first week of lactation, but the effect of training on the behavioural and physiological responses to milking appeared to be influenced by heifer temperament. Svennersten-Sjaunja and Pettersson (2008) found that missed milking negatively influenced cow behaviour, such as less time spent lying and more frequent urinating. The heifers that were allowed to get familiar with the milking parlour before calving had lower heart rates on the first day of lactation than the heifers that had not been familiarized with the new surroundings. According to Schwalm *et al.* (2012) this difference was no longer apparent on day nine of lactation. The heifers habituated quickly to the milking situation including the noise in the milking parlour.

CONCLUSIONS

Farmers, after the introduction of robots, have various problems (substantial deterioration in the quality of milk, non-standard behaviour of dairy cows and many other problems). Therefore, the applied ethological research of robotic milking is highly required.

Generally, the benefits of automation for dairy farm can be seen in improved profitability, animal health, milk quality and farmer lifestyle. A robotic milking is therefore highly topical task. Manual labour is partly replaced by management and control, so the presence of the operator at regular milking times is no longer required. However, the effects of the changeover from conventional parlour to an automatic milking system (RMS) on performance, behaviour and physiological parameters in dairy cows with or without previous experience in RMS milking should be investigated.

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