Veterinarija ir Zootechnika

Volume 77(99)
2020

Veterinarija ir Zootechnika

LITHUANIAN UNIVERSITY OF HEALTH SCIENCES

Volume 77(99)
2020

Aims and Scope

An international scientific journal **"Veterinarija ir Zootechnika"** since 1924 publishes original research and review papers on all aspects of veterinary medicine, food safety and animal sciences. From 1952 to 1994 journal was published under the title: „Acta of Lithuanian Veterinary Academy". After decision of the Research Council of Lithuania under the auspices of the Government of Republic of Lithuania from year 1995 scientific journal „Veterinarija ir Zootechnika" (Vet Med Zoot) was re-established as the Official Organ of the Veterinary Academy (VA) in collaboration with Veterinary Academy, Veterinary Institute, LVA Animal Science Institute, Lithuanian University of Agriculture and Immunology Institute of Vilnius University.

Issues per Year – 4. From 2016 - 2.

The Guide for Authors can be found of the journal's website https://vetzoo.lsmuni.lt/directions-to-authors

Submission of Manuscripts

The manuscripts are registered in the Editorial Office of LSMU VA "Veterinarija ir zootechnika" after filling the Manuscripts Registration Form (*Supplement 1*).
One copy of the manuscript should be addressed to the Editorial Office or e-mailed to the following address: evaldas.slyzius@lsmuni.lt
The manuscripts are refereed (*Supplement 2*) at least by two members of the Editorial Board or appointed referees. The authors are expected to take into consideration the remarks and before the final submission make correction or give motivated explanations of discount of the remarks. The amended paper should be sent back to the Editorial Office in two weeks after the submission of reviews to the author.
If two reviews are negative the article is rejected. If one review is negative, the article should be reviewed by additional referee. If the review of the third referee is positive, the final decision is made by the Editorial board.
The final corrected manuscript should be submitted to the Editorial Board together with the signed letter that authors have approved the paper for release and in agreement with its publication (*Supplement 3*).
The paper should include reference that scientific research has been carried out in accordance with the EU and Lithuanian legal acts.

Publication Information:

„VETERINARIJA ir ZOOTECHNIKA" A scientific journal and the Official Organ of the Veterinary Academy, Lithuanian University of Health Sciences (LSMU VA) ISSN 1392-2130, evaldas.slyzius@lsmuni.lt

© 2020 Lithuanian University of Health Sciences. Production and hosting by MDPI. All rights reserved

Layout Saulius Medžionis
Printed by UAB "Eurispauda", Miško 23, LT-44313 Kaunas, Lithuania. Edition of 10 copies.

Veterinarija ir Zootechnika

LITHUANIAN UNIVERSITY OF HEALTH SCIENCES

Volume 77(99)
2020

EDITORIAL BOARD

Editor in Chief – Dr. Evaldas Šlyžius (Lithuania)
Executive Editor – Dr. Tomas Kupčinskas (Lithuania)

MEMBERS

Prof. Dr. Mindaugas Malakauskas (Lithuania)
Prof. habil. Dr. Saulius Petkevičius (Lithuania)
Prof. Dr. Rasa Želvytė (Lithuania)
Prof. Dr. Rolandas Stankevičius (Lithuania)
Doc. Dr. Birutė Karvelienė (Lithuania)
Dr. Arūnas Rutkauskas (Lithuania)
Dr. Raimundas Mockeliūnas (Lithuania)
Dr. Giedrius Palubinskas (Lithuania)
Dr. Violeta Juškienė (Lithuania)
Dr. Renata Bižienė (Lithuania)

INTERNATIONAL EDITORIAL BOARD

Prof. Thomas Alter (Germany)
Prof. Dr. Albina Aniulienė (Lithuania)
Prof. Dr. Ramūnas Antanaitis (Lithuania)
Prof. Dr. Knud Erik Bach Knudsen (Denmark)
Prof. Dr. Bronius Bakutis (Lithuania)
Prof. Dr. Elena Bartkienė (Lithuania)
Prof. Habil. Dr. Vidmantas Bižokas (Lithuania)
Prof. Anders Miki Bojesen (Denmark)
Assoc. Prof. Dr. Roman Dąbrowski (Poland)
Prof. Habil. Dr. Romas Gružauskas (Lithuania)
Prof. Dr. Ülle Jaakma (Estonia)
Prof. Habil. Dr. Heinz Jeroch (Germany)
Prof. Dr. Vida Juozaitienė (Lithuania)
Dr. Violeta Juškienė (Lithuania)
Prof. Ramunė Kalėdienė (Lithuania)
Assoc. Prof. Dr. Birutė Karvelienė (Lithuania)
Prof. Habil. Dr. Limas Kupčinskas (Lithuania)
Prof. Habil. Dr. Wolfgang Leibold (Germany)
Prof. Vaiva Lesauskaitė (Lithuania)
Prof. Dr. Kazimieras Lukauskas (Lithuania)
Prof. Dr. Alvydas Malakauskas (Lithuania)
Prof. Dr. Darwin K. Murrell (USA)
Prof. Habil. Dr. Dalia Pangonytė (Lithuania)
Prof. Žilvinas Padaiga (Lithuania)
Prof. Dr. Dainius Haroldas Pauža (Lithuania)
Dr. Violeta Razmaitė (Lithuania)
Dr. Smaragda Sotiraki (Greece)
Prof. Arūnas Stankevičius (Lithuania)
Prof. Edgaras Stankevičius (Lithuania)
Prof. Dr. Rimantas Stukas (Lithuania)
Dr. Antanas Šarkinas (Lithuania)
Prof. Dr. Mindaugas Šarkūnas (Lithuania)
Prof. Dr. Loreta Šernienė (Lithuania)
Prof. Dr. Jūratė Šiugždaitė (Lithuania)
Prof. Habil. Dr. Vytas Antanas Tamošiūnas (Lithuania)
Prof. Stig Milan Thamsborg (Denmark)
Habil. Dr. Marian Varady (Slovakia)
Prof. Dr. Gintarė Zaborskienė (Lithuania)
Prof. Dr. Gintaras Zamokas (Lithuania)
Assoc. Prof. Dr. Dailius Zienius (Lithuania)
Prof. Dr. Vytuolis Žilaitis (Lithuania)

Veterinarija ir Zootechnika

LITHUANIAN UNIVERSITY OF HEALTH SCIENCES

Volume 77(99)
Pages 1–63
2020

CONTENTS

The Comparison of Milk Composition and Quality of Dairy Crossbred Simmental and Holstein Cows for One Year. *Kristina de Vitte, Ramutė Mišeikienė, Rolandas Stankevičius, Marius de Vitte* 1

The Effect of Rosemary and Other Natural Food Additives on the Quality of Minced Pork Meat *Sonata Gustienė, Gintarė Zaborskienė, Anita Rokaitytė-Stepankevičienė, Ignė Juknienė, Reda Riešutė* 8

The Effect of Gonadotropin-Releasing Hormone (Gnrh) on Semen Quality and Testosterone Level of Nubian Goats. *Syafruddin Syafruddin, Faris Iryandi, Riska Asria Sa'adatur Rahmi, Husnurrizal Husnurrizal, Teuku Armansyah TR., Budianto Panjaitan, Arman Sayuti, Amalia Sutriana, Dwinna Aliza, Hafizuddin Hafizuddin, Tongku Nizwan Siregar* 16

Characterization of Lactococcus Lactis for Probiotic Properties *in Vitro*. *Kristina Kondrotienė, Neringa Kašėtienė* 22

Re-Emerging Enzootic Bovine Leukosis in A Cattle Farm in Lithuania: A Case Report. *Eglė Rapaliutė, Evelina Stončiūtė, Marius Masiulis, Birutė Karvelienė, Alvydas Malakauskas* 29

The Fatty acid Profile of Intramuscular Fat in the Longissimus Lumborum Muscle from the Bulls of Black-and-White Holstein-Friesian and Their Cross With the Belgian Blue Breed. *Katarzyna Śmiecińska, Dorota Kubiak* 32

Conjunctival Microbiota Susceptibility to Antibacterials in Dogs. *Dovilė Svetikienė, Aidas Grigonis, Gintaras Zamokas, Birutė Karvelienė, Lina Babickaitė, Marija Ivaškienė, Rita Šiugždinienė*............................. 38

Impact of a Diet Supplemented with Exoenzymes and Yeast on Predicted Circadian Rhythm of Reticulorumen pH. *Ignas Šilinskas, Ingrida Monkevičienė, Gintarė Dovydaitienė, Ilma Tapio, Kristina Musayeva, Sigitas Japertas, Antanas Sederevičius, Rasa Želvytė* 45

Influence of Heat Stress and Some Related Physiological Indicators on The Content of Long-Chain Fatty Acids in the Milk of Holstein-Friesian Cows. *Toncho Penev, Nikolina Naydenova, Dimo Dimov, Ivaylo Marinov*.. 51

Investigation of *Mycoplasma* Species in Diseased and Healthy Calves and Heifers in Al-Najaf Province, Iraq. *Khaleel Zainel Khaleel Al-Alo, Ayyed Bajee Hassan Alzwghaibi, Hassan Khalaf Ulaiwi Al-Karagoly, Salim Albukhaty* 59

The Comparison of Milk Composition and Quality of Dairy Crossbred Simmental and Holstein Cows for One Year

Kristina de Vitte[1], Ramutė Mišeikienė[2], Rolandas Stankevičius[1], Marius de Vitte[3]
[1]*Department of Animal Nutrition, Lithuanian University of Health Sciences, Lithuania,*
[2]*Institute of Animal Rearing Technologies, Lithuanian University of Health Sciences, Lithuania,*
[3]*Coventry, England (Coventry University, Priory St, Coventry CV15FB)*

Keywords: *cow, Simmental, Holstein, milk, composition, SCC.*

Abstract. *The research was conducted to ascertain the positive benefits of crossbreeding for the quantitative and qualitative indicators of livestock production. The aim of the research was to perform a comparative analysis of the composition and quality rate of the milk produced by dairy Simmental crossbreeds and Holstein cows during the year.*

The experiment involved the purebred Holstein cows of dairy breed and F1 generation crossbred Holstein x Simmental cows of dual purpose. Twenty cows of each breed were observed for one year. The research revealed that the yield (Simmental crossbreeds: 8,951 l, Holstein 8,665.7 l) during the year was similar in both breeds, but the average annual fat content in the milk (0.32 percent) and protein (0.2percent) was higher in crossbred Simmentals' milk. The lactose content in the milk of both cow breeds differed by 0.075 percent, and no essential differences during the year were observed. The average amount of urea in Holstein cows' milk was 2.65 mg/100 mL, i.e., higher compared with crossbred Simmentals. The quantity of somatic cells in Holstein cows' milk during the year was by 97.85 thousand/mL higher than in the milk of crossbred Simmentals ($P < 0.05$).

Introduction

The period, when rapid freezing of bull semen was introduced 50 years ago, during that period, a new sire was evaluated taking into account the genetic progress of the first lactation daughters. Negative consequences of such an evaluation have long been known; however, it was only in recent years that this fact was taken seriously. It was during that period that the Holstein breed of livestock (Schonmunth, 1963; Malchiodi, 2014) was developed; thus, all the mistakes made during such breeding are best seen and analyzed while studying this particular breed population (Heringstad et al., 2001). Looking for a way out of the situation (Fleischer at al., 2001), two strategies are currently being discussed and used: one suggests including "the functional values of the animal (such as the exterior, life expectancy, hoof wellness, etc.)" into the evaluation of breeding and taking them into account while executing the selection of purebred Holsteins. Another strategy is to exploit the heterosis effect while crossbreeding cattle with other breeds, which has long been known and used for breeding pigs and hens (Hein's et al., 2012; Karamfilov and Nikolov, 2019).

Cattle crossbreeding is of great significance (Konig et al., 2005; Simianer et al., 2006) in Lithuanian dairy farms. The positive effects of crossbreeding make farmers expect higher economic cost-effectiveness by improving cattle health, reproductive characteristics and calf survival rates. In recent years, such problems (cattle health, reproductive characteristic, calf survival rates and so on) have been increasing in the herds of Holstein cows.

Correspondence to Kristina de Vitte, Department of Animal Nutrition, Faculty of Animal Science, Veterinary Academy, Lithuanian University of Health Sciences, Tilžės 18, LT-47181 Kaunas, Lithuania E-mail: kristina.devitte@lsmuni.lt

Simmental breed of dairy cows ranks second in Europe (taking into account the number of livestock as well as the amount of milk and meat produced) after Holstein (SGG, 2018; Perišić et al., 2009). The Global Dairy Simmental Association states that, in some European countries, dairy Simmental breed is dominant, with Germany (about 3.5 million cattle, which accounts for slightly less than 30 percent of all dairy cow population in Germany), Austria (1.6–1.7 million, i.e., about 80 percent of all dairy cow population in Austria) and Serbia (850 thousand, i.e., about 80 percent of all dairy cow population in Serbia) taking the lead. A similar situation also occurs in the Czech Republic, Switzerland and Slovenia (Perišić et al., 2009).

The milk production and milk quality of crossbred cows has been extensively characterized (Heins et al., 2006; Dechow et al., 2007; Prendiville et al., 2010). The aim of the research was to perform a comparative analysis of the composition and quality rate of milk produced by dairy Simmental crossbreeds and Holstein cows during one year of production.

Material and Methods

Twenty Holstein cows and 20 F1 generation crossbred Simmental-Holstein cows were used for the research. All of the cattle were from the second lactation and were calved in November 2017. Livestock was observed and the productivity data were registered and evaluated for one year: from 1 January to 31 December 2018. The cow milk was analyzed once

per month, during the morning and evening milking. The milk quantity was checked and written down during both milkings. Milk samples were taken in an alternating pattern: one month they were taken during the morning, and the following month –they were taken during the evening. Milk indicators were rated using the records accounts of a controlled herd of cattle from 2016–2018. The contents of the milk (fat, protein, lactose and urea) and the indicators somatic cell count in the milk were graded by PE "Pieno tyrimai". Fat, protein, lactose and urea amounts were analyzed using the mid-infrared detector LactoScopeFTIR. The somatic cell count was rated using a Somascope detector, which operates using the flow cytometry principle. The influence of different breeds on profitableness was evaluated using the checks for meat sales/purchases and milk price statistical indexes, which are announced every month on the website of the Ministry of Agriculture of the Republic of Lithuania.

The primary collected information was categorized, grouped, analyzed, and presented in the Microsoft Excel 20 program. Mathematical statistics were used to process the obtained research results and interpretation of the obtained data. The paper presents the following statistical indicators: arithmetic mean and its error (SE, standard deviation, minimum (Min), maximum (Max) and statistical reliability indicator – Student's t criterion with a significance level $P < 0.05$: when $P < 0.05$, the differences between the two comparator groups are considered statistically significant at a 95.0% reliability level; when $P > 0.05$, the differences between the groups are considered statistically unreliable (not statistically significant).

Results

In December, second lactation cows that were selected for the research were dry (Fig. 1). Most dairy crossbred Simmentals and Holsteins calved in January, and the rest did it at the beginning of February. In January, the cows of both breeds produced on the average a similar amount of milk ($P = 0.652$; $P > 0.05$), i.e., dairy crossbred Simmentals produced 17.8 ± 5.62 L, and Holsteins produced 15.5 ± 4.47 L (Fig. 1). In February, the average quantity of milk per cow was a few liters greater than in January, i.e., dairy crossbred Simmentals produced 21.1 ± 6.66 L, and Holsteins produced 18.4 ± 5.31 L.

In 6 weeks after calving, the productivity of most cows becomes the highest. This has also been observed during the research. In March, dairy crossbred Simmental cattle produced an average of 33.5 ± 0.60 L of milk (min = 28.4; max = 40.8;), and Holsteins produced an average of 11.1 L less than dairy crossbred Simmentals (i.e., average 24.4 ± 7.04, min = 6.5; max = 40.8;). However, statistically significant differences were not observed ($P = 0.139$; $P > 0.05$). At the end of April, the grazing period was commenced, which helped maintain high yields of livestock. Both breeds produced more than 33 L per cow. Due to prevailing hot weather in May of 2018, the yield per cow decreased significantly and reached a little more than 27 L per cow. Such an average yield remained until August. Most of the observed cows were successfully inseminated at the beginning of lactation; thus, the milk yield decreased. From October to December, the average yield of the dairy crossbred Simmental cows declined, and the lowest was in November, i.e., 17.4 ± 5.52 L. A similar trend was typical of the yield of Holstein cows. However, the lowest amounts of

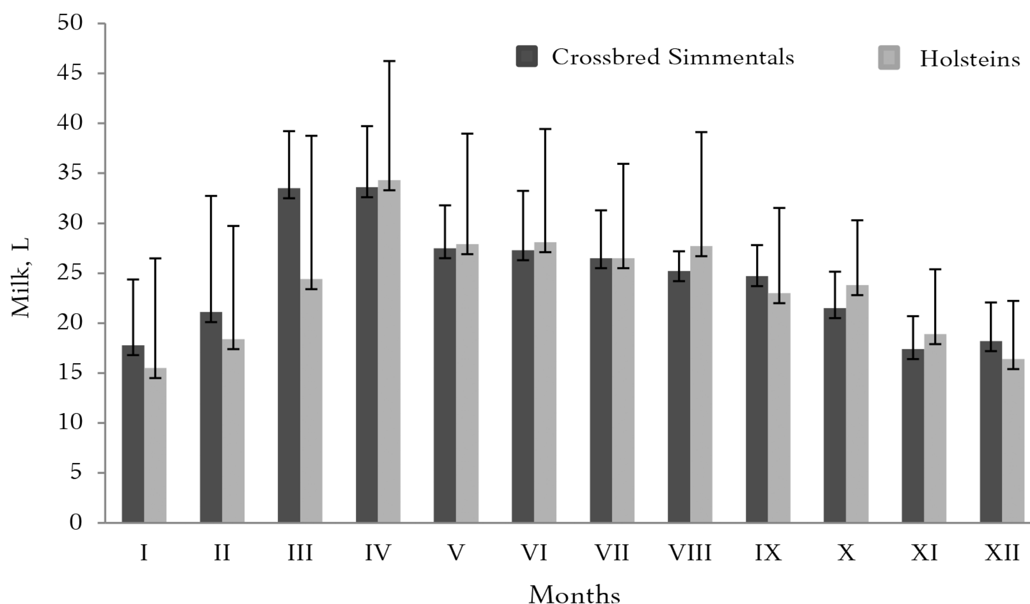

Fig. 1. Milk yield of dairy crossbred Simmental and Holstein cows

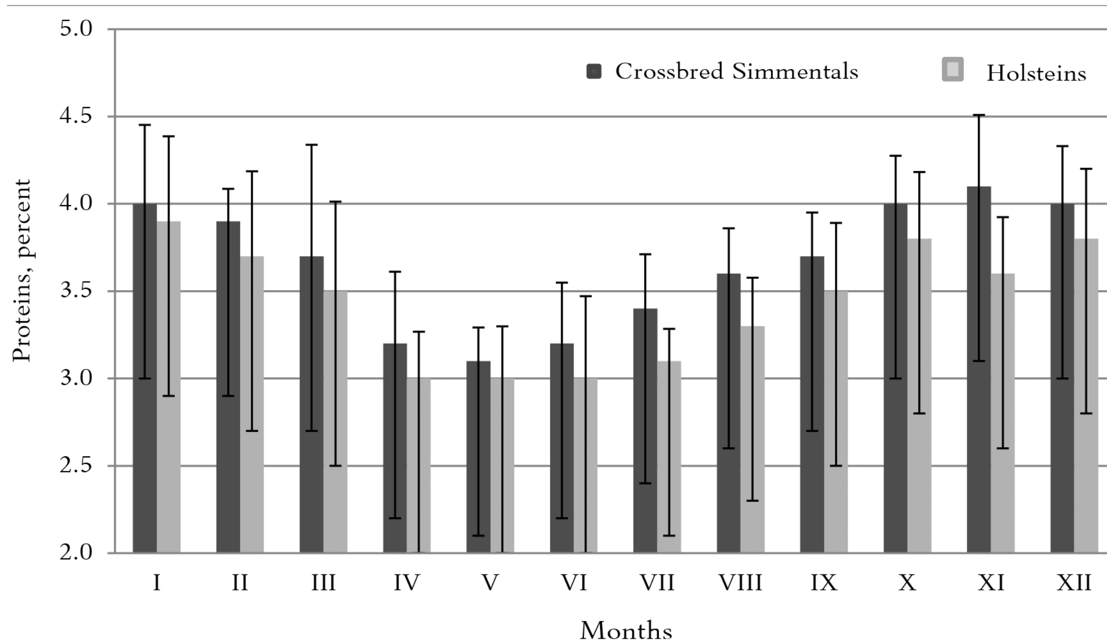

Fig. 2. The protein content in the milk of dairy crossbred Simmental and Holstein cows

milk were produced in December, i.e., an average of 16.4 ± 4.73 L.

The higher protein content in the milk of dairy crossbred Simmental cows, as compared with other varieties, is inherited from generation to generation, and this is one of the reasons for choosing these cows. During the entire period of the research, the protein content in the milk of dairy crossbred Simmental cows was higher than that of Holsteins (Fig. 2). However, statistically significant differences were observed only in November ($P = 0.006$; $P < 0.05$), when the protein content in the milk of dairy crossbred Simmentals was statistically significantly higher by 0.5 percent: for dairy crossbred Simmentals, it was 4.1± 0.41 percent, and for Holsteins, it was 3.6 ± 1.04 percent. During other months, the difference in the protein content in the milk of the two breeds was 0.1–0.3 percent. However, statistically the protein content in the milk of dairy crossbred Simmentals was not higher ($P < 0.05$). The comparison of the tendencies of variation of protein content in the milk of crossbred Simmentals and Holstein cows reveals that the changes in the protein content in the milk of both breeds of cows were very similar, i.e., from January (dairy crossbred Simmentals', with an average of 4.0 ± 0.45 percent, and Holsteins' – 39 ± 0.49 percent) it gradually decreased, while between April and June, it reached the lowest rates (an average of 3.1% to 3.2% in dairy crossbred Simmentals, of, and 3.0% in Holsteins). This was due to the commencement of the grazing period and adaptation to different forage. In June, having stabilized and balanced the changes of forage, the protein content started to grow again, and in October through December it reached the highest average values again, i.e., dairy crossbred Simmentals ranged from 4.0 to 4.1 percent, and Holsteins from 3.6 to 3.8 percent.

The fat content in the milk of the observed cows of both breeds ranged and varied throughout the study unevenly (Fig. 3). The highest average fat content in the milk of dairy crossbred Simmental cows was 5.7 ± 1.01 percent (min = 4.45; max = 7) in March; 5.4 ± 0.75 percent (min = 4.45; max = 6.43) in April; and 5.4 ± 0.86 percent (min = 4.02; max = 6.75) in November. The highest average fat content in the milk of Holstein cows was in January, 5.4 ± 0.90 percent (min = 4.31; max = 6.62) (Fig. 3). The lowest average fat content in the milk of dairy crossbred Simmental cows was observed in October, 4.2 ± 1.19 percent (min = 2.05; max = 6.57), and that of Holsteins in September, 3.9 ± 0.84 percent (min = 2.60; max = 5.43) and 4.2 ± 0.77 percent (min = 2.96; max = 5.20), respectively.

The comparison of the average fat content in the milk of the observed cows of both breeds reveals that throughout the study, except for a few months (January, February and December), the milk fat content of dairy crossbred Simmentals was higher. Key differences were observed in June and July, when the average fat content in the milk of Simmentals was 0.5 percent per piece ($P = 0.017$, $P < 0.05$) and 0.8 percent per piece higher ($P = 0.001$, $P < 0.05$) than in the milk of Holsteins. In March, the average fat content in the milk of dairy crossbred Simmentals was 5.7 ± 1.01 percent (min = 4.45; max = 7.24), and 4.7 ± 1.63 percent in the milk of Holsteins (min = 1.68; max = 6.52), i.e., 1.0 percent higher. However, the difference was not statistically significant.

The amount of lactose in the milk of dairy crossbred Simmentals reached the highest values (4.6 percent) in January through March and December, while in the milk of Holsteins (4.6 ± 1.27 percent), it was highest in December (Fig. 4). The lowest aver-

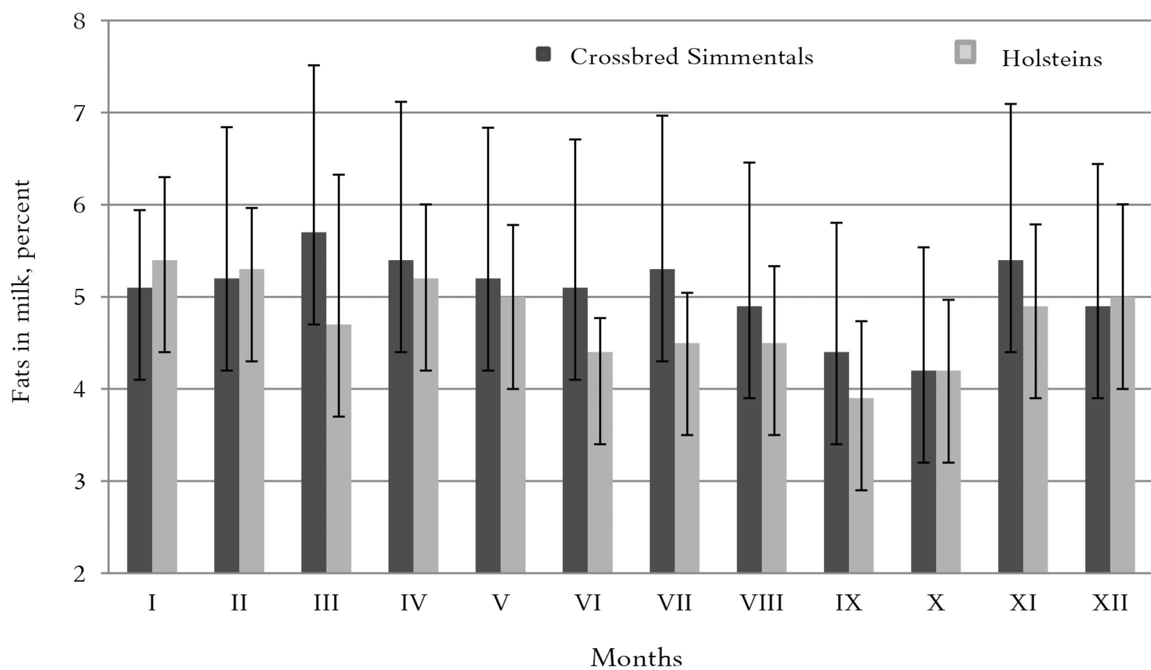

Fig. 3. The fat content in the milk of dairy crossbred Simmental and Holstein cows

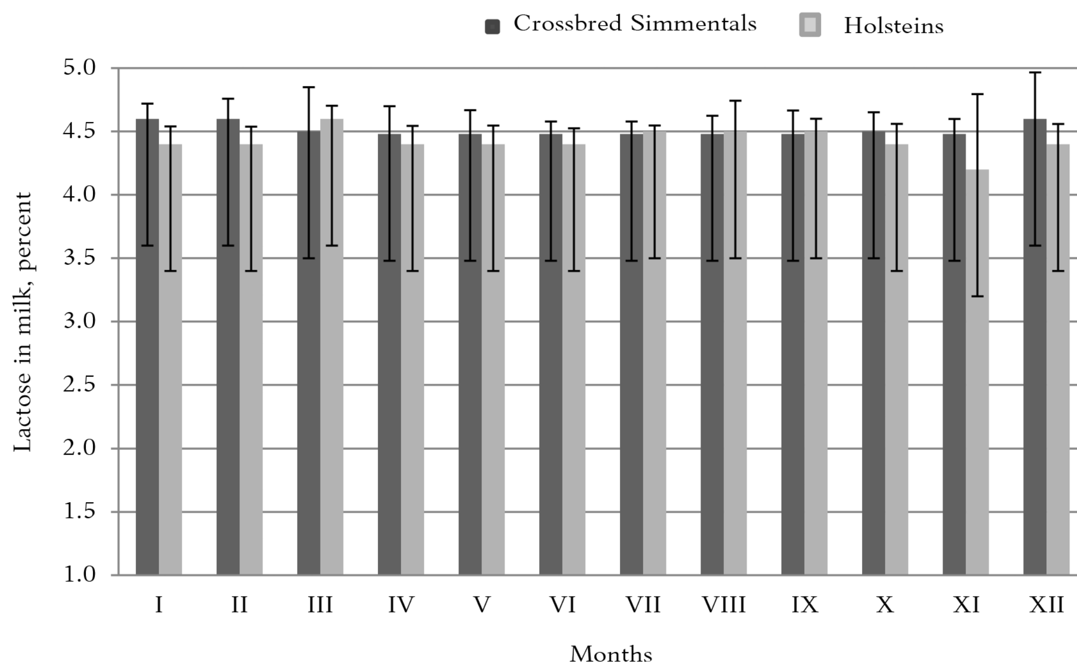

Fig. 4. The amount of lactose in the milk of dairy crossbred Simmental and Holstein cows

age amount of lactose was observed in the milk of Holstein cows in November, i.e., 4.2 ± 1.22 percent (min = 2.48; max = 4.65).

During the entire period of research, the difference in the average amount of lactose in the milk of dairy crossbred Simmental and Holstein cows varied negligibly (0.1–0.3 percent), except in June, when the average lactose content in Simmentals' milk was statistically significantly higher by 0.1 percent than in Holsteins' milk ($P = 0.023$; $P < 0.05$).

The comparison of the amount of urea in the milk throughout the study reveals that usually the average amount of urea in the milk of the Holstein breed was higher than that in the milk of dairy crossbred Simmentals, with the exception of a few months, i.e., January, April and August (Fig. 5). In January, the amount of urea in the milk of dairy crossbred Simmental cows reached 23.2 mg/100 mL, or was statistically significantly greater by 2.1 mg/100 mL than that of Holsteins ($P = 0.049$; $P < 0.05$). In February,

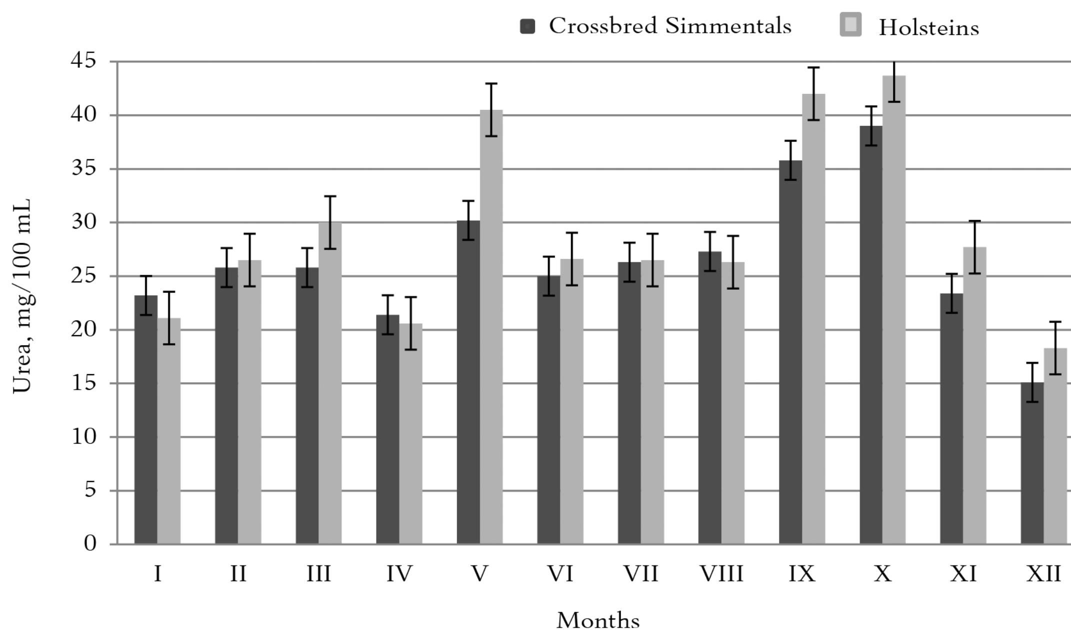

Fig. 5. The amount of urea in the milk of dairy crossbred Simmental and Holstein cows

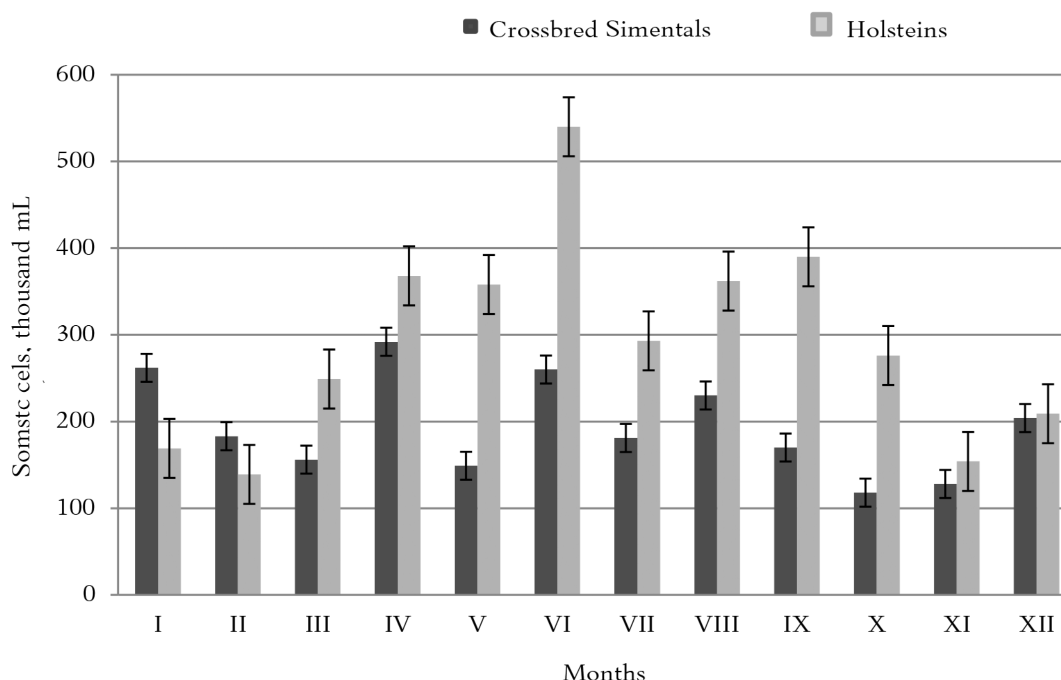

Fig. 6. The somatic cell count in the milk of dairy crossbred Simmental and Holstein cows

the average urea amount in the milk of dairy crossbred Simmentals was 0.7 mg/100 mL, i.e., statistically significantly lower ($P = 0.023$; $P < 0.05$), and in March, it was 4.2 mg/100 mL ($P = 0.028$; $P < 0.05$) lower than in the milk of Holsteins. However, in April, the urea amount in the milk of dairy crossbred Simmental cows was statistically significantly higher by 0.8 mg/100 mL in comparison with the average urea amount in the milk of Holstein cows in the same month ($P = 0.042$; $P < 0.05$).

The study of the established maximal indicators of urea throughout the entire research reveals that the average urea content, more than 40 mg/100 mL, was observed in May, September and October in the milk of Holstein cows, 40.5 ± 8.00 mg/100 mL, 42.0 ± 9.53 mg/100 mL and 43.7 ± 7.98 mg/100 mL, respectively. In comparison with dairy crossbred Simmental cows' milk, urea rates in Holsteins' milk during these months were significantly higher (10.3, 4.2 and 4.7 mg/100 mL, respectively), but the differences were not statistically significant ($P > 0.05$).

The comparison of the average somatic cell counts (SCC) in the milk of dairy crossbred Simmental and Holstein cows reveals that only in January and February the SCC in the milk of dairy crossbred Simmentals was higher than that in Holsteins' milk by 93 and 44 thousand/mL (Fig. 6). During other months, a higher SCC, 5 thousand/mL (December) up to

280 thousand/mL (June) was observed in the milk of Holstein cows. However, during the entire period, the differences in the SCC in the milk of the two breeds of cows were not statistically significant, with the exception of September, when the SCC in the milk of dairy crossbred Simmental cows was significantly lower than that in Holsteins' milk by 220 thousand/mL ($P = 0.034$; $P < 0.05$).

Discussion

During all months, the protein content in the milk of dairy crossbred Simmental cows was by 0.2 percent higher than that in the milk of Holstein cows. The quantity of cow milk depends on many factors: the time of lactation, intensity of feeding, animals' health, genetics, keeping conditions, etc. The research revealed that the milk yields of both breeds were very similar, and no statistically significant differences were observed. The milk yield of Holstein cows averaged 24.5 L per day per cow, while dairy crossbred Simmental cows produced 23.7 L per day per cow ($P > 0.001$). The lowest milk yield of Holstein cows was 8.9 L per cow, and the largest was 59.9 L per cow. The lowest milk yield of dairy crossbred Simmental cows was 12.3 L per cow, the largest was 50.8 L per cow. Similar results were also found in the research conducted by Muller et al. (2005), where the quantity of milk produced by Holstein and dairy crossbred Simmental cows was not significantly different ($P > 0.05$). In Toledo-Alvarado et al. (2017) studies, lower average milk yield of dairy crossbred Simmental cows was observed.

Lower average milk yield of Holstein cows was observed by Pintić et al. (2007), and Abdouli et al. (2008). In the research conducted by Marenjak et al. (2004), the cows of Simmental breed produced more milk than Holstein cows.

During all months, the protein content (3.6 percent) in the milk of dairy crossbred Simmental cows was higher than that in the milk of Holstein cows (3.4 percent). However, substantial differences were observed in November only ($P = 0.006$; $P < 0.05$). Significant differences in milk fat content were observed in June and July, when the average fat content in the milk of dairy crossbred Simmental cows was 0.5 percent ($P = 0.017$, $P < 0.05$) and 0.8 percent respectively higher ($P = 0.001$, $P < 0.05$) than in the milk of Holstein cows. During the year, the average fat content was 4.75 percent in the milk of Holstein cows, and 5.0 percent in the milk of dairy crossbred Simmental cows. In the research conducted by Muller et al. (2005), lower quantities of protein and fat were observed in the milk of Holstein cows than in the milk of dairy crossbred Simmental cows. In the study conducted by Toledo-Alvarado et al (2017), among dairy crossbred Simmental cows, the difference in protein and fat content in the milk has not been established. The study conducted by Pintić et al. (2007) also revealed that no substantial differences in fat and protein content in the milk of Holstein and Simmental breeds were identified. Johnson and Young (2003) as well as Abdouli et al. (2008) estimated that fat and protein content in the milk of Holstein cows was substantially lower as compared with that in the milk of cows of the Simmental breed.

There was no significant difference in the amount of lactose in the milk of both breeds; it ranged from 3.86 percent to 5.59 percent. The lactose content in the milk of dairy crossbred Simmental cows varied little, and the average yearly amount was 4.5 percent, whereas the amount of lactose in the milk of Holstein cows varied greater, with the average of 4.4 percent. Similar results were also obtained in the study conducted by Bendelja et al. (2011).

The average urea amount in Holstein cows' milk was 29.15 mg/100 mL, while in the milk of dairy crossbred Simmental cows it was 26.5 mg/100 mL. Such results fall within the recommended amount of urea in milk defined by most authors (Marenjak et al., 2004). Lower concentration of urea (15.5 mg/100 mL) was found in the milk of Holstein cows in the study conducted by Johnson and Young (2001). However, a 40 percent higher urea amount was observed in Holstein cows' milk as compared with Jersey cows' milk (Rodriguez et al., 1979).

In this study, the quantity of somatic cells in the milk of Holstein cows was statistically significantly higher ($P < 0.001$). The average somatic cell count is 307 thousand/mL. Such an SCC is close to the average somatic cell count characteristic to the breed, which reaches 316 thousand/mL (Hojman et al., 2005). The average SCC in the milk of dairy crossbred Simmental cows was 234 thousand/mL. The study conducted by Toledo-Alvarado et al. (2017) revealed that the somatic cell count was higher in the milk of Holstein cows as compared with the milk of dairy crossbred Simmental cows.

Conclusions

Milk yield during the year was similar in both breeds: Simmental crossbreeds produced 8,951 L, and Holstein cows produced 8,665.7 L. Average annual fat and protein content, 0.32 and 0.2 percent, respectively, was higher in crossbred Simmentals' milk, while the average amount of urea in Holstein cows' milk was 2.65 mg/100 mL, i.e., higher in comparison with crossbred Simmentals. Significant differences were found in November – Simmental crossbreeds: 4.1 ± 0.41 percent, Holstein 3.6 ± 1.04 percent ($P < 0.05$)). Average annual fat content in the milk (0.32 percent) was higher in crossbred Simmentals' milk. Significant differences were found in June and July (Simmental crossbreeds' 0.5 percent ($P = 0.017$; $P < 0.05$) and 0.8 percent higher ($P = 0.001$; $P < 0.05$) some Holstein cows). Lactose content in the milk of both cow breeds differed by 0.075 percent, and no essential differences during the year were observed ($P > 0.05$). The average amount of

urea in Holstein cows' milk was 2.65 mg/100 mL, i.e., higher in comparison with crossbred Simmentals (both stocks were fed the same). Statistically significant differences were found in January (23.2 mg /100 mL) (P = 0.049; P < 0.05) and April (0.8 mg/100 mL) (P = 0.042; P < 0.05), the milk urea content was higher in the milk of dairy Simental crossbred cows than in Holstein cows. The mean urea levels in milk of Simmental crossbreeds in February (0.7 mg/100 mL) (P = 0.023; P < 0.05) and March (4.2 mg/100 mL) (P = 0.028; P < 0.05), the quantity was significantly lower than that of the Holstein. These results suggest a conclusion that dairy crossbred Simmentals use nutrients better for production. The quantity of SCC in Holstein cows' milk during the year was by about 100 thousand/mL higher than in the milk of crossbred Simmentals. In September, the somatic cell count in milk of crossbreed Simmentals was 220 thousand/mL, statistically lower than in Holsteins' milk (P = 0.034; P < 0.05).

References

1. Abdouli H., Rekik B., Haddad - Boubaker A. 2008. Non - nutritional factors associated with milk urea concentrations under Mediterranean conditions. World Journal of Agricultural Sciences. 4. P.183-188.
2. Bendelja D., Prpić Z., Mikulec N., Ivkić Z., Havranek1 J., Antunac N. 2011. Milk urea concentration in cows. Mljekarstvo. 61.1. P. 45-55.
3. Dechow, C. D., G. W. Rogers, J. B. Cooper, M. I. Phelps, and A. L. Mosholder. 2007. Milk, fat, protein, somatic cell score, and days open among Holstein, Brown Swiss and their crosses. J. Dairy Sci. 90:3542–3549.
4. Fleischer P., Metzner M., Beyerbach M., Hoedemaker, Klee W. The relationship between milk yield and the incidence of some diseases in dairy cows. J. Dairy Sci., 84 (2001), pp. 2025-2035
5. Heins, B. J., and L. B. Hansen. 2012. Short communication: Fertility, somatic cell score, and production of Normande × Holstein, Mont-béliarde × Holstein, and Scandinavian Red × Holstein crossbreds versus pure Holsteins during their first 5 lactations. J. Dairy Sci. 95:918–924.
6. Heins, B. J., L. B. Hansen, and A. J. Seykora. 2006. Production of pure Holsteins versus crossbreds of Holstein with Normandie, Montbeliarde, and Scandinavian Red. J. Dairy Sci. 89:2799–2804.
7. Heringstad B., Rekaya R., Gianola G., Klemetsdal G., K.A. 2001.WeigelBayesian analysis of liability of clinical mastitis in Norwegian cattle with a threshold model: Effects of data sampling method and model specification. J. Dairy Sci., 84, pp. 2337-2346
8. Hojman D., Gips M., Ezra E. 2005. Association between live body weight and milk urea concentration in Holstein cows. Journal of Dairy Science. 88. P. 580-584.
9. Johnson, R.G., Young, A.J. 2003. The association between milk urea nitrogen and DHI production variables in Western commercial dairy herds. J of Dairy Sci. 86. P. 3008-3015.
10. Karamfilov S. and Nikolov V.2019. First lactation milk production of cows of the Simmental breed reared in Bulgaria. Bulgarian Journal of Agriculture Science, 25(2). pp.363-369.
11. König S., Dietl G., Raeder I., Swalve H. H. 2005. Genetic relationships for dairy performance between large-scale and small-scale farm conditions. J. Dairy Sci., 88, pp. 4087-4096
12. Malchiodi F., Cecchinato A., Penasa M., Cipolat-Gotet C. and Bittante G. 2014. Milk quality, coagulation properties and curd firmness modeling of pure breed Holsteins and first, and second-generation crossbred cows from Swedish red, Montbeliarde and Brown Swiss bulls. Journal of Diary Science, 97 (7), pp. 4530-4541.
13. Marenjak T., Poljičak-Milas N., Stojević Z. 2004. Svrha određivanja koncentracije ureje u kravljem mlijeku. Praxis veterinaria. 52. P. 233-241.
14. Muller C.J.C., Botha J.A., Cloete S.W.P., Potgieter J.P. 2005. Preliminary results on the comparative of primiparous Holstein and fleckvieh x Holstein dairy cows. P. 3.
15. Perišić P., Skalicki Z., Petrović M. M., Bogdanović V., Ružić - Muslić D. 2009. Simmental cattle breed in different production systems. P.8.
16. Prendiville, R., K. M. Pierce, and F. Buckley. 2010. A comparison between Holstein-Friesian and Jersey dairy cows and their F1 cross with regard to milk yield, somatic cell score, mastitis, and milking characteristics under grazing conditions. J. Dairy Sci. 93:2741–2750.
17. Pintić N., Poljak F., Dakić A., Blažek D., Jelen T., Pintić V. 2007. Kvantitativni pokazatelji kakvoće mlijeka i hranidbeni status krava simentalske i holstein pasmine Potkalničkog kraja. Krmiva. 49. (2) P. 79-88.
18. Rodriguez L.A., Stallings C.C, Herbein J. H, McGilliard M.L, Diurnal 1979. Variation in Milk and Plasma Urea Nitrogen in Holstein and Jersey Cows in Response to Degradable Dietary Protein and Added Fat. Department of Dairy Science, Virginia Polytechnic Institute and State University, Blacksburg 24061-0315 P. 80 – 90.
19. Schönmuth G. 1963. Zur Züchtung eines milchbetonten Zweinutzungsrindes mit hohem Milchfett- und Eiweißgehalt und bestem Euter Arch. Anim. Breed. pp. 79-92
20. Simiane H., Weigend S., RathStrategische D. 2006. Ansätze zur Erhaltung und Nutzung genetischer Diversität innerhalb und zwischen Nutztierpopulationen Agrarspectrum. pp. 33-50
21. Toledo - Alvarado H, Cecchinato A, Bittante G. 2017. Fertility traits of Holstein, Brown Swiss, Simmental, and Alpine Grey cows are differently affected by herd productivity and milk yield of individual cows. Department of Agronomy, Food, Natural Resources, Animals and Environment (DAFNAE), University of Padova P. 30 - 35.
22. Young, A. 2001. Milk urea nitrogen. Utah State University extension. P. 6.
23. SGG (2018). http://swissgenetics.com/en/genetics/breed-specific - Information.

Received 28 January 2020
Accepted 4 June 2020

The Effect of Rosemary and Other Natural Food Additives on the Quality of Minced Pork Meat

Sonata Gustienė[1], Gintarė Zaborskienė[1], Anita Rokaitytė-Stepankevičienė[1], Ignė Juknienė[1], Reda Riešutė[2]

[1]Department of Food Safety and Quality, Faculty of Veterinary Medicine, Veterinary Academy Lithuanian University of Health Sciences, Lithuania

[2]Food Institute, Kaunas University of Technology, Lithuania

Key words: *minced meat, bacteria cultures, antioxidant activity, fatty acids, peroxides.*

Summary. *The demand for high-quality safe meat products has been increasing. In the study, the effects of chosen antioxidants (rosemary, coenzyme Q10, taurine, and creatine) on minced pork meat quality were evaluated. The pork was obtained from Lithuanian producer X without any added water or spices. The study assessed the effects of selected antioxidants in combination with bacterial cultures (Staphylococcus xylosus and Pediococcus pentosaceus) on microbial, physical, chemical and sensory indicators as well. Testing was done on day 1 to day 5 of the study at a 2–4°C temperature, under aerobic conditions. Samples with mixtures of rosemary and other natural additives reduced the pH, amount of yeast and mould, and amount of biogenic amines, acids, and peroxides compared with control. The visual quality of the meat was also improved; therefore, rosemary, in addition to other natural additives, could be used in preparation of new meat products.*

Introduction

Meat and meat products occupy one of the most important places in nutrition in terms of their high-quality proteins, essential amino acids, vitamins, minerals and other nutrients (Zhang et al., 2010). Therefore, more and more consumers and scientists have recently been discussing the quality and healthiness of meat products. Producers are being urged by consumers to create more natural, safer and higher-quality meat products. Consumers want products to be made of high-quality raw materials using fewer artificial preservatives that do not cause allergies or increase the sensitivity of the body (Mariutti et al., 2011). Due to synthetic antioxidants that cause toxic effects, there is an increased demand for natural antioxidants by consumers and the meat industry (Fernandes et al., 2018). Currently, there is a high interest in natural antioxidants as many plants (and their extracts) that have antioxidant activity and at the same time health benefits are usually used as spices (Fernandes et al., 2017; Poojary et al., 2017; Putnik et al., 2017). Anti-oxidative properties of spices are particularly interesting because of their impact on oxidative stress suppression which could lead to the prevention of inflammatory, cardiovascular, neurodegenerative diseases and cancer (Srinivasan et al., 2016). Natural antioxidants in products postpone oxidative lipid degradation, improve food nutritional value, quality and replace synthetic antioxidants (Fadda et al., 2010). They also have a positive impact on health by protecting biologically important cellular structures, such as membrane lipids and proteins, DNA, from reactive oxygen attacks (Su et al., 2007). Phenolic compounds are major components of most plant extracts that determine the anti-oxidative properties (Munekata et al., 2017). They act as free radical inhibitors and as chelating agents for metal ions, e.g., iron and copper (Chan et al., 2014). Natural food additives are also useful in attempting to reduce the amount of biogenic amines (Lee et al., 2018; Wang et al., 2019), which are toxic nitrogenous compounds and are often used as indicators of food spoilage (Fiddes et al., 2014; Li et al., 2014). Biogenic amines can also be converted into nitrosamines, which are known to be carcinogens (National Toxicology Program, Department of Health and Human Services, 2016), in the presence of nitrite and nitrate. (De Mey et al., 2017). Therefore, eliminating biogenic amines as much as possible seems to be an important challenge.

Another alternative that can ensure effectiveness of the technological process and safety meat products, are bacterial cultures which usually contain lactic acid bacteria. Lactic acid bacteria are naturally found in many food products during fermentation. Studies have shown that using bacterial cultures improved meat product colour and taste properties (Bourdichon et al., 2012). In addition to being non-toxic, bacteria can be digested with proteases and thus have no effect on the gut microbiota (Zendo, 2013).

In minced pork meat production, bacterial cultures are usually not used, but in order to ensure the quality of the product during the marketing period without preservatives, the use of bacterial cultures is being looked into.

The aim of this research was to evaluate the effect of added antioxidants and bacterial culture mixtures in minced pork meat on microbiological and physical-chemical factors in order to select the most appropriate supplement.

Gustienė, Department of Food Safety and Quality, Faculty of Veterinary Medicine, Veterinary Academy Lithuanian University of Health Sciences, Tilžės 18, LT-47181 Kaunas, Lithuania
E-mail: sonata.gustiene@lsmuni.lt

Materials and Methods. Pork tenderloin and notch were purchased from the Lithuanian manufacturer X without added water or spices. At the beginning of the technological process, the meat was minced using a sterile 3 mm sieve. The minced meat was divided into 0.5 kg batches and kept under aerobic conditions at +4°C temperature. Chemical substances and their selected concentration relied on a literature review of previous studies and analyses. During the study, the effect on microbiological, physicochemical and organoleptic characteristics of selected antioxidants with bacterial cultures (*S. xylosus, P. pentosaceus*) in minced pork was evaluated. The tests were carried out on day 1 to day 5, under aerobic conditions, at 2–4°C temperature.

Combinations of compounds used in study were as follows: group 1 samples – rosemary extract 0.05% + taurine 0.01% + bacterial cultures; group 2 samples – rosemary extract 0.05% + creatine 0.01% + bacterial cultures;group 3 samples – rosemary extract 0.05 % + coenzyme Q10 0.05 % + bacterial cultures; group 4 samples – rosemary extract 0.05 % + bacterial cultures; group 5 samples – coenzyme Q10 0.05% + bacterial cultures; group 6 samples – creatine 0.01 % + coenzyme Q10 0.05% + bacterial cultures; and group 7 samples – control without tested chemicals and bacterial cultures.

The microbiological and physical-chemical analysis of the samples were performed after 24, 72 and 120 h of storage.

pH measurement

The pH of the sample was measured according to the standard method for determination of meat pH: EN ISO 2917:2002 (EN ISO, 2002). pH measurements were carried out using a PP-15 pH-meter (Sartorius Professional meter for pH Measurement, Germany).

Microbiological analysis

Samples of 10 g were taken at random for each sample and aseptically weighed into a sterile stomacher bag with 90 mL of sterile buffered peptone water 0.1% (*m/V*) (REF 611014, Liofilchem, Italy) and homogenized for 1 min in a model 400 Stomacher (Seward Medical, London, UK). The total number of beta-glucuronidase-positive *Escherichia coli* (*E. coli*) with a positive beta-glucuronidase reaction was determined using a pour plate technique on the tryptone, bile, and glucuronide medium, incubating for 24 h at 44 °C in accordance with LST ISO 16649-2:2002 (LST ISO, 2002). The number of colonies of yeasts and moulds were determined on a Dichloran Rose agar (DRBC; REF 17147, Sigma-Aldrich, Italy) with chloramphenicol selective supernatant, incubating for 120 h at 25 °C in accordance with LST ISO 21527-1:2008 (LST ISO, 2008). The total count of mesophilic bacteria was determined on plate count agar (PCA, Sigma-Aldrich, Merck) after incubation at 30 °C for 72 h in accordance with LST EN ISO 4833-1:2013 (LST ISO, 2013). After incubation, colonies were counted according to LST ISO 7218:2007 (LST ISO, 2007). The microbiological data were transformed into the logarithm of the number of colony forming units (CFU/g).

Biogenic amines content

A reversed-phase high-performance liquid chromatography (RP-HPLC) method was used for the quantitative analysis of biogenic amines: tryptamine, putrescine, cadaverine, histamine, tyramine, and spermine. The whole cured samples were cut into small pieces and mashed mechanically using a homogenizer (Moulinex Masterchef 20, Nieune, France). Biogenic amine content was extracted from the homogenized sample with 0.4 mol/L perchloric acid. The derivatization of samples was carried out using the modified methodology of Ben-Gigirey et al. (2000). The extract was derivatized for 45 min with dansyl chloride (5-dimethylaminonaphtalene-1-sulfonylchloride) solution in acetone at 40 °C. The samples were filtered through a 0.45 μm membrane filter (Millipore Co., Bedford, MA, USA), and 10 μL was injected into a chromatographic system (Aligent 1200 Series, Waldbronn, Germany). The analysis was performed using LiChro column CART® 95 125-4 (Merck, Darmstadt, Germany).

Free radical scavenging activity (DPPH assay)

The method used by Takao et al. (1994) was adopted with suitable modifications from Kumarasamy et al. (2007). DPPH (2, 2-dephenyl-1-picrylhydrazyl) (8.0 mg) was dissolved in MeOH (100.0 mL) to obtain a concentration of 80 μg/mL. Serial dilutions were carried out with the stock solution (1mg/mL) of the samples extract. Solutions (2.0 mL each) were then mixed with DPPH (2.0 mL) and left to stand for 30 min for any reaction to occur, and the absorbance was measured at 515 nm.

Acid value

Acid value of the extracted lipids was determined according to EN ISO 660:2009 (EN ISO, 2009) procedure.

Peroxide value

Peroxide value of the studied lipids was determined according to EN ISO 3960:2010 (EN ISO, 2010) iodometric method and was presented as meqv O_2/kg lipids (Latimer GWJr).

Fatty acids content

The amount of fatty acids was determined by the method of gas chromatography using a flame ionization detector. For the analysis of fatty acids, the samples were prepared according to the standard EN ISO 12966-2:2011 (EN ISO, 2011). Fatty acids were methylated using anhydrous KOH methanol solu-

tion. Chromatographic analysis of fatty acid methyl esters was performed using gas chromatograph Shimadzu GC – 17A, using BPX – 70, 120 m column, following the methodology determined in EN ISO 15304:2003/AC:2005 2 (EN ISO, 2005). The fatty acid methyl esters (FAME) were identified by comparison of each retention time with the Supelco 37 Component FAME mix (catalog No-47885-U).

Colour determination

Meat surface colour was measured using a reflectance spectrophotometer (Minolta CM-2002; Osaka, Japan). Parameters measured in the reflection mode were L^*, a^* and b^* (corresponding to brightness, redness and yellow coordinates according to the CIE scale) (C.I.E. 1978).

Statistical Analysis of the Data

Data were analyzed using the SPSS 20.0 software (SPSS Inc., Chicago, Illinois, USA). Differences between data were evaluated by tGhe analysis of the variance method (one-way ANOVA) with a significant level of $p < 0.05$. Multiple comparisons were estimated by the Fisher's least significant difference method, and the Dunnet test was applied when the control group was present. The Student *t* test was used to determine average values of indicators, standard deviations and linear correlations. The correlation was considered reliable when $p < 0.05$.

Results and Discussion

The initial pH of minced pork was 5.35 ± 0.02. In all groups of examined samples, the pH was evenly reduced (Table 1). The results showed a statistically significant difference between control and samples with bacterial cultures after 72 and 120 h ($p < 0.05$). The lower pH values in inoculated samples could be related to the fact that the inoculation of the bacterial cultures resulted in a stronger acidification during the storage process.

The number of *E. coli*, which depends on the quality of the raw material and hygiene conditions during the process, was similar after 24 h in all tested samples: from 0.64 ± 0.25 to 1.78 ± 0.24 $\log_{10}$ CFU/g (Table 2). After 3 days, a decrease in *E. coli* was observed in all samples with bacterial cultures compared with control ($p < 0.05$). This outcome is in agreement with those reported previously by other authors (Lorenzo et al., 2014; Dominguez et al., 2016) who observed lower *E. coli* counts in inoculated samples compared with a control batch. The decrease on *E. coli* counts could be explained by the pH decrease and the growth of lactic acid bacteria (Lorenzo et al., 2014).

The total number of mesophilic aerobic bacteria found in Group 3 (5.23 ± 0.31 $\log_{10}$ CFU/g) and Group 4 (5.14 ± 0.50 $\log_{10}$ CFU/g) samples of minced pork, containing rosemary extract, showed a statistically significant reduction in the total bacterial count compared with the rest of the samples ($p < 0.05$) after 24 h. After 72 h, the total number of bacteria increased significantly in all the samples compared with the samples after 24 h, and there were no statistically significant differences compared with the control group ($p > 0.05$).

A slight increase in the number of yeasts and moulds was observed from 24 h during storage. There were no significant differences between control and samples with added antioxidants and bacterial cultures ($p > 0.05$).

Biogenic amine accumulation (expressed as mg/kg) is shown in Table 3. Generally, tyramine, cadaverine and putrescine are the main amines found in meat products (Dominguez et al., 2016).

In our study, the main biogenic amine in the samples was spermine (between 14 and 20 mg/kg), followed by cadaverine (about 11 mg/kg), spermidine (about 10 mg/kg) and tyramine (between 6 and 8 mg/kg). The total amount of biogenic amines after 24 h was statistically higher in Group 6 (98.55 ± 3.67 mg/kg) and control (101.66 ± 4.63 mg/kg) samples ($p < 0.05$). Bio-

Table 1. Effects of antioxidants and bacterial cultures on acidity (pH) during storage in minced pork

Samples	t (storage)/hours		
	After 24	After72	After120
Group 1	5.46 ± 0.09[a]	4.97 ± 0.02[a]	5.36 ± 0.07[a]
Group 2	5.51 ± 0.02[a]	5.08 ± 0.05[a]	5.30 ± 0.02[a]
Group 3	5.49 ± 0.08[a]	5.05 ± 0.09[a]	5.21 ± 0.03[a]
Group 4	5.50 ± 0.05[a]	5.09 ± 0.05[a]	5.23 ± 0.08[a]
Group 5	5.45 ± 0.06[a]	5.18 ± 0.05[a]	5.47 ± 0.06[a]
Group 6	5.42 ± 0.05[a]	5.11 ± 0.06[a]	5.35 ± 0.10[a]
Control	5.39 ± 0.02[a]	5.48 ± 0.05[b]	5.77 ± 0.06[b]

Results are expressed as mean value ± standard deviation. Different letters in superscript indicate significant differences between the samples in the same row. Group 1 samples – rosemary extract 0.05 % + taurine 0.01 % + bacterial cultures; Group 2 samples – rosemary extract 0.05 % + creatine 0.01 % + bacterial cultures; Group 3 samples – rosemary extract 0.05 % + coenzyme Q10 0.05 % + bacterial cultures; Group 4 samples – rosemary extract 0.05 % + bacterial cultures; Group 5 samples – coenzyme Q10 0.05 % + bacterial cultures; Group 6 samples – creatine 0.01 % + coenzyme Q10 0.05 % + bacterial cultures; and Group 7 samples – control without tested chemicals and bacterial cultures.
a, b – Means in the same column with different letters are significantly different, $P < 0.05$

Table 2. Effects of antioxidants and bacterial cultures on microbiological profile (log_{10} CFU/g) during storage in minced pork

Microbiological profile	Samples	t (storage)/hours		
		After 24	After 72	After 120
Escherichia coli	Group 1	1.05 ± 0.32[a]	0.04 ± 0.01[a]	0.00 ± 0.00[a]
	Group 2	0.64 ± 0.25[a]	0.00 ± 0.00[a]	0.00 ± 0.00[a]
	Group 3	0.77 ± 0.18[a]	0.00 ± 0.00[a]	0.00 ± 0.00[a]
	Group 4	0.82 ± 0.27[b]	00.00 ± 0.00[a]	0.00 ± 0.00[a]
	Group 5	1.65 ± 0.19[b]	0.21 ± 0.02[b]	0.00 ± 0.00[a]
	Group 6	1.75 ± 0.11[b]	0.14 ± 0.03[b]	0.00 ± 0.00[a]
	Control	1.78 ± 0.24[b]	1.35 ± 0.29[c]	0.66 ± 0.14[b]
Total mesophilic aerobic bacteria	Group 1	6.32 ± 0.44 [a]	6.50 ± 0.32 [a]	7.63 ± 0.44 [a]
	Group 2	6.41 ± 0.69 [a]	6.31 ± 0.24 [a]	7.55 ± 0.30 [a]
	Group 3	5.23 ± 0.31 [b]	6.17 ± 0.37 [a]	7.10 ± 0.25 [a]
	Group 4	5.14 ± 0.50 [b]	6.10 ± 0.41 [a]	7.13 ± 0.72 [a]
	Group 5	6.54 ± 0.67 [a]	6.41 ± 0.62 [a]	7.55 ± 0.41 [a]
	Group 6	6.76 ± 0.36 [a]	6.89 ± 0.57 [a]	7.82 ± 0.40 [a]
	Control	6.78 ± 0.51 [a]	6.53 ± 0.74 [a]	8.03 ± 0.62 [a]
Sum of yeasts and moulds	Group 1	1.11 ± 0.29 [a]	2.06 ± 0.29 [a]	2.52 ± 0.29 [a]
	Group 2	1.85 ± 0.12 [a]	2.19± 0.34 [a]	2.36 ± 0.31[a]
	Group 3	1.10 ± 0.21 [a]	1.63 ± 0.26 [a]	2.36 ± 0.31[a]
	Group 4	1.23 ± 0.29 [a]	1.87 ± 0.31[a]	2.06 ± 0.17 [a]
	Group 5	1.05 ± 0.21 [a]	1.94 ± 0.35 [a]	2.25 ± 0.25 [a]
	Group 6	1.95 ± 0.34 [a]	2.11 ± 0.21[a]	2.15 ± 0.41 [a]
	Control	1.13 ± 0.29 [a]	2.21 ± 0.29 [a]	2.75 ± 0.23 [a]

Results are expressed as mean value ± standard deviation. Different letters in superscript indicate significant differences between the samples in the same row. Sample abbreviations are given in Table 1.
a, b – Means in the same column with different letters are significantly different, $p < 0.05$.

Table 3. Effects of antioxidants and bacterial cultures on total biogenic amine content (mg/kg) and biogenic amine index during storage in minced pork

Biogenic amines	Samples	t(storage)/hours		
		After 24	After 72	After 120
Total biogenic amine content	Group 1	53.82 ± 6.51 [a]	95.72 ± 3.24 [a]	187.30 ± 5.18 [ab]
	Group 2	60.05 ± 5.38 [a]	97.27 ± 4.48 [a]	176.02 ± 7.60 [a]
	Group 3	32.23 ± 3.59 [c]	69.34 ± 8.61 [b]	109.49 ± 6.31 [b]
	Group 4	20.34 ± 2.55 [c]	51.04 ± 5.79 [b]	114.81 ± 5.17 [b]
	Group 5	51.81 ± 1.38 [a]	94.85 ± 9.72 [b]	158.49 ± 8.28 [b]
	Group 6	98.55 ± 3.67 [b]	159.27 ± 4.33 [a]	180.32 ± 9.07 [a]
	Control	101.66 ± 4.63 [b]	194.57 ± 7.41 [a]	201.72 ± 7.65 [a]
Biogenic amine index	Group 1	46.62 ± 2.51 [a]	85.93 ± 6.82 [a]	163.52 ± 8.36 [a]
	Group 2	50.95 ± 4.26 [a]	80.87 ± 5.37 [a]	161.21 ± 7.22 [a]
	Group 3	27.93 ± 2.39 [b]	58.23 ± 6.05 [ab]	97.39 ± 7.31 [ab]
	Group 4	17.96 ± 3.15 [b]	47.03 ± 5.39 [a]	100.05 ± 8.25 [a]
	Group 5	45.95 ± 2.62 [a]	79.75 ± 4.38 [a]	150.47 ± 5.11 [a]
	Group 6	85.58 ± 6.33 [c]	106.35 ± 4.36 [a]	173.86 ± 9.50 [a]
	Control	86.16 ± 3.08 [c]	170.91 ± 3.25 [a]	184.61 ± 8.74 [a]

Results are expressed as mean value ± standard deviation. Different letters in superscript indicate significant difference between the samples in the same row. Sample abbreviations are given in Table 1.
a, b, c – Means in the same column with different letters are significantly different, $p < 0.05$.

genic amines increased in all samples during storage, but a significantly smaller amount was found in Group 3 sample (109.49 ± 6.31 mg/kg) and IV (114.81 ± 5.17 mg/kg) after 120 h ($p < 0.05$). Biogenic amines form from the enzymatic decarboxylation of amino acids by microbial enzymes (Li et al., 2014). Therefore, the higher the amount of spoilage microorganisms, the higher the amount of biogenic amines.

The same trend of increase can be seen in the control group when taking into account the biogenic amine index as well. The biogenic amine index (BAI) takes histamine, putrescine, cadaverine, and tyramine into account and is the sum of these four biogenic amines (Özogul and Özogul, 2019). Cadaverine and putrescine are precursors to N-nitrosopyrrolidine, a carcinogenic nitrosamine, as well as other nitrosamines (Drabik-Markiewicz et al., 2011). High levels of tyramine and histamine have many adverse effects on human health, such as high blood pressure caused by tyramine, and allergy-like reactions due to histamine (Latorre-Moratalla et al., 2017). Because of these effects to human health, it is ideal to have a low biogenic amine index. After 120 h, a statistically lower BAI, compared with the rest of the samples, was only found in samples 3 and 4 ($p < 0.05$). Taking into account both the results of the total amount of biogenic amines and the BAI, it seems that samples 3 and 4 with rosemary extract and bacterial cultures were the best at reducing biogenic amines and are a valuable additive to prevent the accumulation of high levels of toxic biogenic amines.

Research has shown that rosemary extract has high antioxidant activity and can be used in the meat industry. In the tested minced pork, the highest DPPH free radical binding was in samples with a mixture of rosemary extract and coenzyme Q10 (42.26 ± 0.03%) (Table 4). The study showed that the number of acids and the number of peroxides were effectively reduced compared with control samples. This ensures the reduction in oxidation of lipids during the production and storage of minced meat. In a sample of minced pork with rosemary extract (Group 4), a statistically significant ($p < 0.05$) low number of acids (1.77 ± 0.01 mg KOH/g) and statistically significant ($p < 0.05$) low acidity based on oleic acid (0.89 ± 0.01%) were found. Antioxidants have a positive effect on the reduction of acid and peroxides during minced pork storage.

Interest in the composition of fatty acids in meat has increased due to the need to find ways to produce healthier meat and meat products. That is to produce meat and its products with a higher content of polyunsaturated fatty acids compared with saturated fatty acids, and the adjusted ratio between Omega-6 and Omega-3 fatty acids (Wood et al., 2004). The supplementation of minced pork with antioxidants and bacterial cultures additives in the samples tested was different (Table 5).

Table 4. Effects of antioxidants and bacterial cultures on antioxidant activity during storage in minced pork

Indicators	Samples	t (storage)/hours		
		After 24	After 72	After 120
DPPH (%)	Group 1	38.51 ± 6.82 ab	24.86 ± 2.47 ab	53.52 ± 8.36 ab
	Group 2	40.15 ± 3.71 ab	26.80 ± 4.21 b	56.21 ± 7.22 ab
	Group 3	42.26 ± 4.36 a	38.11 ± 6.33 b	43.86 ± 9.50 a
	Group 4	40.21 ± 3.25 a	27.13 ± 3.06 ab	44.61 ± 8.74 a
	Group 5	32.07 ± 4.38 b	26.15 ± 2.62 a	30.47 ± 5.11 b
	Group 6	39.18 ± 6.05 ab	31.35 ± 2.31 a	37.39 ± 7.31 ab
	Control	32.03 ± 5.39 a	29.15 ± 3.16 a	37.05 ± 8.25 a
Acid value (mgKOH-kg)	Group 1	0.68 ± 0.21 a	2.52 ± 0.34a	2.81 ± 0.21a
	Group 2	1.23 ± 0.17 a	3.09 ± 0.26 a	3.20 ± 0.16 a
	Group 3	0.94 ± 0.25 a	1.96 ± 0.20 a	2.14 ± 0.17 a
	Group 4	1.11 ± 0.16 b	1.68 ± 0.31 b	2.32 ± 0.25 b
	Group 5	2.02 ± 0.32 b	2.81 ± 0.15 b	3.21 ± 0.13 b
	Group 6	1.45 ± 0.19 b	2.73 ± 0.28 b	2.92 ± 0.15 b
	Control	1.95 ± 0.22 b	2.91 ± 0.34 ab	3.22 ± 0.37 ab
Peroxide value (meqvO2-kg)	Group 1	0.73 ± 0.04 ab	0.82 ± 0.02 ab	1.52 ± 0.06 ab
	Group 2	0.84 ± 0.03 ab	0.91 ± 0.03 b	1.21 ± 0.02 ab
	Group 3	0.67 ± 0.03 a	0.69 ± 0.02 b	0.86 ± 0.05 a
	Group 4	0.51 ± 0.01 a	0.62 ± 0.01 ab	0.91 ± 0.04 a
	Group 5	0.91 ± 0.03 b	0.97 ± 0.01 a	1.47 ± 0.11 b
	Group 6	0.56 ± 0.02 ab	0.65 ± 0.02 a	1.39 ± 0.03 ab
	Control	0.73 ± 0.03 a	0.88 ± 0.03 a	1.05 ± 0.05 a

Results are expressed as mean value ± standard deviation. Different letters in superscript indicate significant differences between the samples in the same row. Sample abbreviations are given in Table 1.
a, b – Means in the same column with different letters are significantly different, $p < 0.05$.

Table 5. Effects of antioxidants and bacterial cultures on fatty acid content during storage (after 72 hours) in minced pork

	Samples						
	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Control
Saturated fatty acids	39.59 ± 0.03 ^{a}	35.59 ± 0.03 ^{b}	40.63 ± 0.06 ^{b}	43.54 ± 0.05 ^{a}	37.25 ± 0.08 ^{b}	40.00 ± 0.03 ^{ab}	40.09 ± 0.24 ^{b}
Monounsaturated fatty acids	45.63 ± 0.45 ^{a}	52.60 ± 0.04 ^{a}	48.57 ± 0.40 ^{a}	43.93 ± 0.03 ^{b}	44.21 ± 0.10 ^{b}	44.08 ± 0.00 ^{a}	42.58 ± 0.15 ^{ab}
Polyunsaturated fatty acids	11.30 ± 0.03 ^{a}	9.71 ± 0.11 ^{ab}	7.21 ± 0.03 ^{b}	11.47 ± 0.85 ^{a}	13.74 ± 0.05 ^{a}	11.99 ± 0.14 ^{b}	11.01 ± 0.16 ^{a}
Trans isomers	2.84 ± 0.06 ^{a}	1.24 ± 0.28 ^{b}	2.35 ± 0.03 ^{a}	2.65 ± 0.02 ^{a}	2.75 ± 0.18 ^{ab}	2.88 ± 0.16 ^{a}	2.90 ± 0.25 ^{b}
Omega-3 fatty acids	0.55 ± 0.03 ^{b}	0.65 ± 0.01 ^{b}	0.76 ± 0.52 ^{b}	0.43 ± 0.01 ^{a}	0.25 ± 0.21 ^{b}	0.23 ± 0.17 ^{b}	0.55 ± 0.23 ^{b}
Omega-6 fatty acids	9.91 ± 0.01 ^{b}	8.62 ± 0.03 ^{b}	6.09 ± 0.40 ^{a}	10.58 ± 0.08 ^{a}	12.71 ± 0.14 ^{a}	11.25 ± 0.18 ^{a}	9.57 ± 0.45 ^{a}
Ratio omega-6 / omega-3 fatty acids	17.89 ± 0.63 ^{a}	13.19 ± 0.02 ^{a}	7.98 ± 1.18 ^{a}	24.72 ± 0.12 ^{ab}	51.04 ± 0.09 ^{b}	49.57 ± 0.26 ^{b}	17.50 ± 0.38 ^{b}

Results are expressed as mean value ± standard deviation. Different letters in superscript indicate significant differences between the samples in the same row. Sample abbreviations are given in Table 1.
a, b – Means in the same column with different letters are significantly different, $p < 0.05$.

Most of the saturated fatty acids were found in Group 4 (43.54 ± 0.05) and the least in Group 2 (35.5 ± 0.03). The highest amount of monounsaturated fatty acids was found in sample 2 of minced pork (52.60 ± 0.04). Most polyunsaturated fatty acids were found in samples with coenzyme Q10. Fatty acid trans-isomers were detected in very small amounts in all tested samples. Omega-3 fatty acids were detected in similar amounts in minced pork from 0.23 ± 0.17 up to 0.76 ± 0.52. From this study, we can conclude that the largest ratio was in Group 2 with rosemary extract and creatine.

Color is a very important factor in the quality of meat. Consumer perception of the product is often influenced by the color of the product. Color gives meat not only an aesthetic appearance, but also is related to qualities. Minced pork redness (a^*) increased ($p < 0.05$) in all samples after day 1 (Table 6).

Muscle color refers to the amount of protein myoglobin and its form in the muscle. Due to the effect of oxygen, myoglobin (purple) turns into oximyoglobin, which gives the meat a reddish-red color. At the end of the minced pork storage, we found a statistically significant dif-

Table 6. Influence of antioxidants on colour change during storage in minced pork

Colour	Samples	t (storage)/hours		
		After 24	After 72	After 120
L *	Group 1	44.43± 2.21ab	41.73 ± 2.11ab	39.68 ± 2.14ab
	Group 2	42.80 ± 1.09b	41.93 ± 1.41a	40.03 ± 1.19b
	Group 3	45.60 ± 1.67b	42.24 ± 2.11a	32.87 ± 1.71b
	Group 4	44.84 ± 1.48ab	39.71 ± 1.38ab	36.10 ± 1.12ab
	Group 5	45.60 ± 1.67a	42.24 ± 2.11a	40.20 ± 1.89a
	Group 6	42.76 ± 1.48b	34.61 ± 1.17a	31.46 ± 1.17a
	Control	39.11 ± 2.14b	35.63 ± 1.18a	33.65 ± 1.85b
a *	Group 1	12.77 ± 1.39b	15.18 ± 1.31a	14.15 ± 0.31ab
	Group 2	10.21 ± 1.21b	11.82 ± 1.01a	10.80 ± 1.88a
	Group 3	11.08 ± 0.96b	13.80 ± 0.92a	12.25 ± 1.92b
	Group 4	12.81 ± 1.85b	15.20 ± 0.88a	13.74 ± 1.18ab
	Group 5	12.23 ± 1.45ab	13.81 ± 0.58ab	11.63 ± 1.10ab
	Group 6	10.02 ± 0.96ab	13.70 ± 0.92ab	12.45 ± 0.83ab
	Control	11.08 ± 0.96b	12.80 ± 0.92a	12.70 ± 0.92a
b *	Group 1	11.94 ± 1.68a	10.98 ± 1.52b	9.47 ± 1.49b
	Group 2	12.23 ± 1.45b	11.81 ± 0.53a	10.82 ± 0.39a
	Group 3	10.25 ± 2.11a	9.31 ± 0.32a	8.96 ± 0.67b
	Group 4	11.27 ± 1.57b	9.92 ± 1.61a	9.92 ± 1.61a
	Group 5	14.01 ± 1.32ab	10.98 ± 1.52ab	8.74 ± 0.74ab
	Group 6	12.26 ± 1.52b	11.25 ± 1.06ab	10.23 ± 1.06b
	Control	11.94 ± 1.68a	10.98 ± 1.51b	9.34 ± 1.87a

Results are expressed as mean value ± standard deviation. Different letters in superscript indicate significant differences between the samples in the same row. Sample abbreviations are given in Table 1.
a, b – Means in the same column with different letters are significantly different, $p < 0.05$.

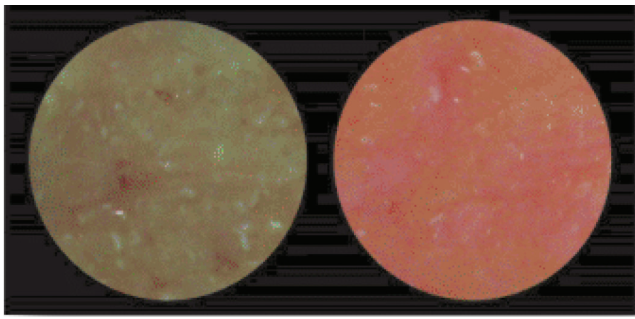

Fig. 1. The effect of rosemary extract on minced pork after 72 hours

ference between the samples with the added antioxidants and the control ($p < 0.05$). The effect of rosemary extract on minced pork (Fig. 1) was also noticeable as the extract had an effect on red color stability.

The yellow color (b^*) of minced pork decreased rapidly, but there was no statistically significant difference between control and samples with added bacterial cultures ($p > 0.05$).

Conclusion

The results of the study confirmed that rosemary extract has antioxidant activity and can be used in the development of new food products. Rosemary paired with coenzyme Q10 reduced pH the most out of the studied samples, and yeasts and moulds were best reduced in a sample with rosemary extract. The additives also reduced the amount of acids and peroxides, as well as the amount of biogenic amines. The effect of rosemary extract on minced pork color was also noticeable as it affected the pink color stability. Because minced pork gets pink, which is the preferred indicator, consumers appreciate it as the product looks more attractive.

The effect of each antioxidant is different when used in different meat matrices, e.g., the antioxidant effect of coenzyme Q10 in minced pork is significantly higher in mixtures with other antioxidants, and may not work, e.g., in marinated thighs. Therefore, not only the chosen concentration but also the complex components of the meat product influence the effect of the antioxidant on the safety and quality of the meat product. To conclude, rosemary extract, with not only strong antibacterial but also antioxidative properties, can be used as an antioxidant.

Acknowledgments

We thank the Lithuanian Science Foundation for financial support for this research.

References

1. Ben-Gigirey B., Vieites Baptista de Sousa J.M., Villa T.G., Barros-Velazquez J. Characterization of biogenic amine-producing Stenotrophomonas maltophilia strains isolated from white muscle of fresh and frozen albacore tuna. International Journal of Food Microbiology. 2000. T. 57. P. 19-31.
2. Bourdichon F., Casaregola S., Farrokh C., Frisvad J.C., Gerds M.L., Hammes W.P., et al. Food fermentations: Microorganisms with technological beneficial use. International Journal of Food Microbiology. 2012. T. 154. P. 87-97.
3. Büyükköroğlu G., Dora D.D., Özdemir F., Hızel C.. Omics Technologies and Bio-Engineering. USA, Academic Press, 2018. P. 317-351.
4. Camo J., Beltrán A., Roncalés P. Extension of the display life of lamb with an antioxidant active packaging. Meat Science. 2008. T. 80. P. 1086-1091.
5. Chan K.W., Khong N.M.H., Iqbal S., Ch'ng S.E., Younas U., Babji A.S. Cinnamon bark deodorised aqueous extract as potential natural antioxidant in meat emulsion system: a comparative study with synthetic and natural food antioxidants. Journal of Food Science & Technology. 2014. T. 51. P. 3269–3276.
6. Cleveland J., Montville T.J., Nes I.F., Chikindas M.L. Bacteriocins: safe, natural antimicrobials for food preservation. International Journal of Food Microbiology. 2001. T. 71. P. 1-20.
7. De Mey E., De Maere H., Paelinck H., Fraeye I. Volatile N-nitrosamines in meat products: Potential precursors, influence of processing, and mitigation strategies. Critical Reviews in Food Science and Nutrition. 2017. T. 57. N. 13. P. 2909-2923.
8. Deegan L.H., Cotter P.D., Hill C., Ross P. Bacteriocins: Biological tools for bio-preservation and shelf-life extension. International Dairy Journal. 2006. T. 16. P. 1058-1071.
9. Dominguez R., Munekata P.E., Agregan R., Lorenzo J.M. Effect of commercial starter cultures on free amino acid, biogenic amine and free fatty acid contents in dry-cured foal sausage. LWT-Food Science and Technology. 2016. T.74. N. 2. P. 47-53.
10. Drabik-Markiewicz G., Dejaegher B., De Mey E., Kowalska T., Paelinck H., Vander Heyden Y. Influence of putrescine, cadaverine, spermidine or spermine on the formation of N-nitrosamine in heated cured pork meat. Food Chemistry. 2011. T. 126. N. 4. P. 1539-1545.
11. Draper J.V., Kaber D.B., Usher J.M. Telepresence. Human factors. 1998. T. 40. N. 3. P. 354-375.
12. EN ISO 12966-2:2011. Animal and vegetable fats and oils. Gas chromatography of fatty acid methyl esters. Part 2: Preparation of methyl esters of fatty acids. Geneva, Switzerland. P. 15.
13. EN ISO 15304:2003/AC:2005. Animal and vegetable fats and oils. Determination of the content of trans fatty acid isomers of vegetable fats and oils. Gas chromatographic method. Geneva, Switzerland. 2005. P. 20.
14. EN ISO 3960:2010. Animal and vegetable fats and oils. Determination of peroxide value. Iodometric (visual) endpoint determination. Geneva, Switzerland. 2010. P. 10.
15. EN ISO 660:2009. Animal and vegetable fats and oils. Determination of acid value and acidity. Geneva, Switzerland. 2009. P. 9.
16. Fadda S., López C., Vignolo G. Role of lactic acid bacteria during meat conditioning and fermentation: Peptides generated as sensorial and hygienic biomarkers. Meat Science. 2010. T. 86. P. 66–79.
17. Fasseas M.K., Mountzouris K.C., Tarantilis P.A., Polissiou M., Zervas G. Antioxidant activity in meat treated with oregano and sage essential oils. Food Chemistry. 2008. T. 106. P. 1188-1194.
18. Fernandes M., Mutch D., Leri F., Fernandes M.F., Mutch D.M., Leri F. The Relationship between Fatty Acids and Different Depression-Related Brain Regions, and Their Potential Role as Biomarkers of Response to Antidepressants. Nutrients. 2017. T. 9. N. 3 P. 298.
19. Fernandes R.P.P., Trindade M.A., Lorenzo J.M., de Melo M.P. Assessment of the stability of sheep sausages with the addition of different concentrations of Origanum vulgare extract during storage. Meat Science. 2018. T. 137. P. 244–257.
20. Fiddes L.K., Chang J., Yan N. Electrochemical detection of biogenic amines during food spoilage using an integrated sensing RFID tag. Sensors and Actuators B: Chemical. 2014. T. 202. P. 1298-1304.

21. Gálvez A., Abriouel H., Lucas López R., Ben Omar N. Bacteriocin-based strategies for food biopreservation. International Journal of Food Microbiology. 2007. T. 120. P. 51-70.
22. IBM SPSS Statistics for Windows, v. 20.0, IBM Corp., Armonk, NY, USA; Available from: https://www.ibm.com/us-en/?lnk=m.
23. Kumarasamy Y., Byres M., Cox P.J., Jaspars M., Nahar L., Sarker S.D. Screening seeds of some Scottish plants for free radical scavenging activity. Phytother. 2007. T. 21. P. 615-621.
24. Latorre-Moratalla M.L., Comas-Basté O., Bover-Cid S., Vidal-Carou M.C. Tyramine and histamine risk assessment related to consumption of dry fermented sausages by the Spanish population. Food and Chemical Toxicology. 2017. T. 99. P. 78-85.
25. Lee JY., Kim YG., Her JY., Kim M. K., Lee KG. Reduction of biogenic amine contents in fermented soybean paste using food additives. LWT. 2018. T. 98. P. 470-476.
26. Li M., Zhao G., Zhang Q., Gao X., Huang X., Sun L. Formation of biogenic amines and growth of spoilage-related microorganisms in pork stored under different packaging conditions applying PCA. 2014. Meat Science. T. 96. N. 2. P. 843-848.
27. Lorenzo J.M., Gómez M., Fonseca S. Effect of commercial starter cultures on physicochemical characteristics, microbial counts and free fatty acid composition of dry-cured foal sausage. Food Control. 2014. T. 46. N.1. P. 382-389.
28. LST EN ISO 4833-1:2013. Microbiology of the food chain. Horizontal method for the enumeration of microorganisms. Part 1: Colony count at 30 degrees C by the pour plate technique. Vilnius, Lithuania. 2013. P. 9.
29. LST ISO 16649-2:2002. Microbiology of food and animal feeding stuffs. Horizontal method for the enumeration of beta-glucuronidase-positive Escherichia coli. Part 2: Colony-count technique at 44 degrees C using 5-bromo-4-chloro-3-indolyl beta-D-glucuronide. Vilnius, Lithuania. 2001. P. 8.
30. LST ISO 21527-1:2008. Microbiology of food and animal feeding stuffs. Horizontal method for the enumeration of yeasts and moulds. Part 1: Colony count technique in products with water activity greater than 0,95. Vilnius, Lithuania. 2008. P. 8.
31. ISO 7218:2007. Microbiology of food and animal feeding stuffs - General requirements and guidance for microbiological examinations. Vilnius, Lithuania. 2007. P. 8.
32. LST ISO 2917:2002. Meat and meat products. Determination of pH. Reference method. Vilnius, Lithuania. 2002. P. 6.
33. Maqsood S., Benjakul S. Comparative studies of four different phenolic compounds on in vitro antioxidative activity and the preventive effect on lipid oxidation of fish oil emulsion and fish mince. Food chemistry. 2010. T. 119. P. 123-132.
34. Maqsood S., Benjakul S. Preventive effect of tannic acid in combination with modified atmospheric packaging on the quality losses of the refrigerated ground beef. Food Control. 2010. T. 21. P. 1282-1290.
35. Marino M., Bersani C., Comi G. Impedance measurements to study the antimicrobial activity of essential oils from Lamiaceae and Compositae. International Journal of Food Microbiology. 2001. T. 67. N. 3 P. 187-195.
36. Mariutti L.R.B., Nogueira G.C., Bragagnolo N. Lipid and Cholesterol Oxidation in Chicken Meat Are Inhibited by Sage but Not by Garlic. Journal of Food Science. 2011. T. 76. N. 6. P. 909-915.
37. Munekata P.E.S., Domínguez R., Campagnol P.C.B., Franco D., Trindade M.A., Lorenzo J.M. Effect of natural antioxidants on physicochemical properties and lipid stability of pork liver pâté manufactured with healthy oils during refrigerated storage. Journal of Food Science & Technology. 2017. T. 54. P. 4324–4334.
38. National Toxicology Program, Department of Health and Human Services. Report on Carcinogens. Ataskaita. 2016. Internete: https://ntp.niehs.nih.gov/ntp/roc/content/profiles/nitrosamines.pdf
39. Özogul Y. and Özogul F. Biogenic Amines Formation, Toxicity, Regulations in Food. In: Biogenic Amines in Food: Analysis, Occurrence and Toxicity (Ed. Saad B., Tofalo R. London, UK. 2019. P. 1-17.
40. Poojary M.M., Dellarosa N., Roohinejad S., Koubaa M., Tylewicz U., Gómez-Galindo F., et al. Influence of Innovative Processing on γ-Aminobutyric Acid (GABA) Contents in Plant Food Materials. Comprehensive Reviews in Food Science and Food Safety. 2017. T. 16. P. 895-905.
41. Putnik P, Bursać Kovačević D, Režek Jambrak A, Barba F, Cravotto G, Binello A, et al. Innovative "Green" and Novel Strategies for the Extraction of Bioactive Added Value Compounds from Citrus Wastes—A Review. Molecules. 2017. T. 22. N. 5. P. 680.
42. Srinivasan K. Spices and Flavoring Crops: Uses and Health Effects. In: Encyclopedia of Food and Health (Caballero B, Finglas PM, Toldrá F). Mysore, India. 2016. P. 98–105.
43. Su L., Yin J-J., Charles D., Zhou K., Moore J., Yu L. Total phenolic contents, chelating capacities, and radical-scavenging properties of black peppercorn, nutmeg, rosehip, cinnamon and oregano leaf. Food Chemistry. 2007. T. 100. P. 990-997.
44. Takao T., Kitatani F., Watanabe N., Yagi A., Sakata K. A Simple Screening Method for Antioxidants and Isolation of Several Antioxidants Produced by Marine Bacteria from Fish and Shellfish. Bioscience, Biotechnology, and Biochemistry. 1994. T. 58. N. 10. P. 1780-1783.
45. Wang J., Fang J., Wei L., Zhang Y., Deng H., Guo Y., Hu C., Meng Y. Decrease of microbial community diversity, biogenic amines formation, and lipid oxidation by phloretin in Atlantic salmon fillets. LWT. 2019. T. 101. P. 419-426.
46. Wojdyło A., Oszmian´ski J., Oszmian´ski O., Czemerys R. Antioxidant activity and phenolic compounds in 32 selected herbs. Food Chemistry. 2007. T. 105. P. 940-949.
47. Wood J., Richardson R., Nute G., Fisher A., Campo M., Kasapidou E, et al. Effects of fatty acids on meat quality: a review. Meat Science. 2004. T. 66. P. 21-32.
48. Woraprayote W., Malila Y., Sorapukdee S., Swetwiwathana A., Benjakul S., Visessanguan W. Bacteriocins from lactic acid bacteria and their applications in meat and meat products. Meat Science. 2016. T. 120. P. 118-132.
49. Zendo T. Screening and Characterization of Novel Bacteriocins from Lactic Acid Bacteria. Bioscience, Biotechnology, and Biochemistry. 2013. T. 77. P. 893-899.
50. Zhang W., Xiao S., Samaraweera H., Lee E.J., Ahn D.U. Improving functional value of meat products. Meat Science. 2010. T. 86. N. 1. P. 15-31.

Received 5 April 2020
Accepted 28 April 2020

The Effect of Gonadotropin-Releasing Hormone (Gnrh) on Semen Quality and Testosterone Level of Nubian Goats

Syafruddin Syafruddin[1], Faris Iryandi[2], Riska Asria Sa'adatur Rahmi[2], Husnurrizal Husnurrizal[3], Teuku Armansyah TR.[4], Budianto Panjaitan[1], Arman Sayuti[1], Amalia Sutriana[4], Dwinna Aliza[5], Hafizuddin Hafizuddin[3*], Tongku Nizwan Siregar[3]

[1]*Laboratory of Clinic, Faculty of Veterinary Medicine, Universitas Syiah Kuala, Indonesia*
[2]*Study Program of Veterinary Medicine, Faculty of Veterinary Medicine Universitas Syiah Kuala, Indonesia*
[3]*Laboratory of Reproduction, Faculty of Veterinary Medicine Universitas Syiah Kuala, Indonesia*
[4]*Laboratory of Pharmacology, Faculty of Veterinary Medicine Universitas Syiah Kuala, Indonesia*
[5]*Laboratory of Pathology, Faculty of Veterinary Medicine Universitas Syiah Kuala, Indonesia*

Keywords: *GnRH, Nubian buck, Reproductive performance, Spermatozoa quality, Testosterone*

Abstract. *This research aimed to determine the effect of gonadotropin-releasing hormone (GnRH) in improving semen quality and hormone testosterone in Nubian goats. The experimental design was a 3x3 Latin square with the experimental animals that received a physiological NaCl injection as a control (T0), 50 μg GnRH (T1), and 100 μg GnRH (T2). The semen samples were collected using an artificial vagina at 24 hours after treatment, and then semen characteristics were evaluated both macroscopically and microscopically. Subsequently, the blood samples were collected at 60 minutes after the injection of GnRH for the analysis of testosterone concentration using the enzyme-linked immunosorbent assay (ELISA) method. The observations showed a cream coloration with thick consistency in the semen collected from all treatment groups. In addition, the average (±SD) of semen volume (mL), concentration (10^6), motility (%), viability (%), abnormality (%) of spermatozoa and testosterone levels (ng/mL) in the T0 vs T1 vs T2 groups were 1.8 ± 0.52 vs 1.5 ± 0.70 vs 2.6 ± 1.63 ($P > 0.05$), 807 ± 409.98 vs 895 ± 509.73 vs 1,215 ± 270.14 ($P > 0.05$), 37.00 ± 0.333 vs 34.00 ± 0.309 vs 65.00 ± 0.110 ($P > 0.05$), 63.00 ± 0.144 vs 59.00 ± 0.121 vs 57.00 ± 0.145 ($P < 0.05$), 33.00 ± 0.382 vs 15.00 ± 0.199 vs 7.00 ± 0.040 ($P > 0.05$), 13.16 ± 9.37 vs 28.13 ± 1.21 vs 33.13 ± 2.30 ($P < 0.05$), respectively. It was concluded that GnRH treatment is capable of reducing spermatozoa abnormalities and increasing the testosterone concentrations of Nubian goats.*

Introduction

Semen and hormonal concentrations are important indicators of the quality of male reproduction (Novita et al. 2006). Several studies have shown a lower quality of spermatozoa in Nubian goats, compared with the Peranakan Etawah (PE), and similar to the Kacang goat. Hastono et al. (2013) reported that spermatozoa of Anglo-Nubian goats show the creamy coloration, thick consistency, semen volume of 0.43 ± 0.05 mL, concentration of 2.77 ± 0.27 million/mL, motility ++, and viability of 58.30 ± 27.30%, while the PE spermatozoa present with a creamy-yellow color, watery consistency, semen volume of 0.86 ± 0.40 mL, concentration of 3.10 ± 0.57 million/mL, motility +/+++++, and viability of 75.98 ± 4.61. In Kacang goats, the number of spermatozoa was 2.763 ± 395.0 million/mL with motility of 3.7 (Armansyah et al. 2018). The testosterone levels of Nubian goats at puberty range around 5.4 ng/mL (Souza et al. 2011), which is relatively lower than recorded for Kacang goats. Armansyah et al. (2018) reported an average testosterone level of Kacang goats in the control, prostaglandin F2 alpha, and seminal vesicle extracts treatment groups to be 10.27 ± 5.42, 18.51 ± 19.46, and 29.57 ± 12.96 ng/mL, respectively.

Improving the quality of Nubian semen involves processes that increase the volume and concentration of spermatozoa, increasing the gonadotropin-releasing hormone (GnRH) circulation in blood. This compound has a decapeptide in the structure and is synthesized in the arcuate nucleus of the hypothalamus (Suparman and Suparman 2016) with a basic function of regulating adenohipofisa activity, subsequently stimulating the release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (Isnaini and Wahjuningsih 2014). FSH is the main gonadotropin hormone affiliated with the process of spermatogenesis (Akmal et al. 2015), while LH, which is secreted by the anterior pituitary, controls the development of germ cells. Collectively, they have been associated with the release of androgens by interstitial cells, necessary in the production of mature sperm, and also in the stimulation of Leydig cells to secrete testosterone (Andalusia et al. 2008), which assist in the formation of spermatozoa (Hasbi and Gustina 2018).

The GnRH hormone is often used to increase the reproductive capacity of male animals, including buffalo (Sajjad et al. 2007), PE goats (Hamdan 1999),

Correspondence to Hafizuddin Hafizuddin, Laboratory of Reproduction, Faculty of Veterinary Medicine, Universitas Syiah Kuala, Jalan Tgk Hasan Krueng Kalee No. 4, Darussalam, Banda Aceh 23111, Indonesia. E-mail: hafizuddin_umar@unsyiah.ac.id

and sheep (Azawi et al. 2012; Kiyma et al. 2000). However, there has been no research on its application for similar a purpose on Nubian goats. Azawi et al. (2012) stated that the breed and area of origin influence the reproductive activity of sheep. Therefore, research is needed to determine the effect of GnRH in improving the quality of semen and the hormone testosterone in Nubian goats.

Materials and Methods

This research required the use of three male Nubian goats aged 2–3 years, with a 3 × 3 latin square design. The male goats were raised in individual cages, were fed 3–4 kg of king grass and 0.5–0.7 kg of concentrate daily, and had water consumption *ad libitum*. The experimental animals received physiological NaCl injections as controls (T0), 50 µg gonadorelin (Fertagyl, BV Boxmer, Holland, T1), and 100 µg gonadorelin (T2), and each treatment was performed three times and on alternate weeks. This procedure was conducted according to the instructions of Hamdan (1999), as schematically presented in Table 1.

Research Procedures

Semen collection

Semen was collected using an artificial vagina, previously dried and cleaned alongside the rubber and reservoir tubes, in order to prevent contamination. The sample was collected in one ejaculation/week for three weeks, while the time interval between treatment and collection was in accordance with Azawi et al. (2012).

Semen quality examination

Semen macroscopic examination

After collection, the quality was macroscopically evaluated based on volume, color, consistency, and pH.

Semen microscopic examination

Spermatozoa motility

Spermatozoa motility was evaluated by dripping the sample on a glassslide, then one drop of physiological NaCl was added, followed by observation through a microscope with 40 x 10 magnification. The number of motile sperm was calculated based on the movement, categorized as fast progressive (A), slow progressive (B), circular (C) and fibration (D). The percentage was determined using the following formula:

$$\%\ \text{motility} = \frac{A}{A + B + C + D} \times 100\ \%$$

Spermatozoa concentration

Spermatozoa concentrations were calculated using a haemocytometer pipette and a Neubauer counting chamber, and the semen was sucked up to a scale of 0.5, followed by the addition of a 3% NaCl solution, until 101 was reached. Subsequently, the solution was gently homogenized using an “8” pattern for 2–3 minutes, then a few drops were discarded, followed by another round of homogenization. The sample was filled into the Neubauer counting chamber, and closed with a glass cover. Spermatozoa counting was performed in five chambers, and observed under a microscope with 40 x 10 magnification. The concentration obtained was Y x 5 x 10^6 (Y = the number of spermatozoa in 5 boxes) (Indriani et al. 2013).

Spermatozoa Viability

The examination of viability was performed by introducing one drop of spermatozoa on a glass slide, followed by the addition of one staining eosin drop. A smear preparation was made and fixed on a spritus lamp, and then was evaluated using a microscope of 40 x 10 magnification. The dead cells absorb red pigmentation, while the live spermatozoa tend to not absorb any color, leading to a white appearance. The spermatozoa were then counted and divided by the total visible, and presented as a percentage value (Masir et al. 2017; Padrik et al. 2010).

$$\%\ \text{Live} = \frac{\text{Total of live spermatozoa}}{\text{Total live and dead spermatozoa}} \times 100\%$$

Spermatozoa Abnormalities

This observation was performed by dripping spermatozoa and eosin on the object glass, fixed on a spritus lamp, and observed in a microscope with 40 x 10 magnification (Klimas et al. 2012; Rahmiati et al. 2015). The morphological examination identified deformities that are categorized as primary (small/large head size, double head or double tail, and abnormal head shape) and secondary abnormalities (head rupture, tail breaking at the neck or middle, and folded tail). The minimum spermatozoa observed were 200 cells, and the calculations was conducted using the following formula:

$$\%\ \text{Abnormality} = \frac{\text{Abnormality}}{\text{Abnormality} + \text{Normal}} \times 100\%$$

Blood sampling

A total of 3 mL blood samples without anticoagulants were collected through the jugular vein using a syringe, 60 minutes after administering the GnRH injection (Hamdan 1999). The blood was then placed into a centrifuge tube and left for 60 minutes to freeze, and the serum was subsequently separated from blood objects (clots), followed by centrifugation at 1200 rpm for 10 minutes. The collected serum was transferred into a microtube and stored in the freezer at –20°C (Gholib et al. 2016).

Hormone testosterone analysis

Hormone analysis was performed according to the procedure from the testosterone catalog from DRG

diagnostic (EIA-1559, DRG Instruments GmbH, Germany). Briefly, a standard solution of 0.2 ng/mL to 16 ng/mL was prepared. A total of 25 µL of each standard solution and samples were transferred into the micro-plate well. Subsequently, 200 µL of conjugate enzyme was added and the mixture was incubated for 60 minutes at room temperature, and then the micro-plate was washed three times using a 300 µL washing solution in each well. Furthermore, 200 µL of substrate solution was added to each well and incubated for 15–20 minutes at room temperature, followed by the termination of enzyme reaction with a stop solution of 100 µL 0.5 M H_2SO_4. The absorbance was read using an ELISA reader (Pratomo and Yudi 2016).

Data Analysis

Data resulting from the quality examination of semen and testosterone were analysed using ANOVA followed by the Duncan multiple range test.

Results

The results of volume, concentration, motility, viability and abnormalities examination on Nubian goat spermatozoa in the three treatment groups, including the physiological NaCl group (A), 50 µg GnRH (B), and 100 µg GnRH (C), are presented in Table 2.

Discussion

The color of semen collected from Nubian goats in all treatments was cream with thick consistency. This was in accordance with Ax et al. (2000), who recorded milky white or cream in color of goat semen, while Tambing et al. (2003) reported on the interrelatedness of both characteristics, indicating an association between a thinner semen and paler coloration.

The average semen volume obtained was 1.50–2.60 mL, while the spermatozoa volume at T0, T1, and T2 were 1.80 ± 0.52, 1.50 ± 0.70, and 2.63 ± 1.63 mL ($P>0.05$), respectively. The semen volume tends to increase in goats treated with 100 µg, although no statistically significant effect was recognized. In addition, the semen volume in the 100 µg treatment group was higher than the value obtained with PE goat as reported by Hamdan (1999), who capped at 1.30 mL, using a similar GnRH dose level.

The semen volume was included in the normal category, although Garner and Hafez (2000) reported that a normal range was from 0.80 to 1.20 mL. Hafizuddin et al. (2020) report that semen volume in Anglo-Nubian x PE (Anpera) crossbred goats in the age groups of 24 months, 30 months, 36 months, and more than 48 months were 0.60 ± 0.08 mL, 0.78 ± 0.05 mL, 0.84 ± 0.18 mL, and 0.75 ± 0.03 mL, respectively. Meanwhile, the occurrence of an increase per ejaculation is often associated with the optimal working capacity of the testes and the accessory glands, resulting from the influence of GnRH (Hamdan 1999). However, the variation between high and low volume is affiliated with the frequency of ejaculation, species, age, season, nutrition, libido, and animal condition (Tambing et al. 2003). According to Pamungkas et al. (2008), besides the differences in goat species, the collection method and frequency as well as the age have also been identified as influencing factors.

The result of microscopic semen examination indicated that a spermatozoa concentrations in T0, T1, and T2 were 807 ± 409.98, 895 ± 509.73, and 1.215 ± 270.14 (10^6/mL) ($P > 0.05$), respectively. Numerically, the average concentration tends to increase in the treatment group, with no statistically significant effect. This was possibly due to an elevation in the levels of FSH and testosterone, which have been implicated in spermatogenesis activities of goat (Hamdan 1999). However, the spermatozoa concentration observed in this study (range 807–1.215 (10^6/mL)) was lower than the normal value observed in goat, which is 3.50 x 10^9 to 6.00 x 10^9/mL (Ax et al. 2000). These were lower than the values recorded for PE, as reported by Hamdan (1999), using similar dosages. Husin et al. (2007) reported on the influence of differences in species, as the concentration of spermatozoa was assumed to be due to its genetic quality (Situmorang 2002). This has also been affiliated with variations in the age of the male, which increases up to the 22nd month (Heriyanta et al. 2014).

Spermatozoa motility levels in T0, T1, and T2 were 37.00 ± 0.33, 34.00 ± 0.30, and 65.00 ± 0.11%, respectively, with a quality ranging from 34% to 65% ($P > 0.05$). Meanwhile, the average value tends to increase more in those treated with GnRH at a dose of 100 µg, although no statistically significant effect was observed. The minimal elevation in motility was possibly related to the increased concentration of spermatozoa per mL of semen (Hamdan 1999), which is known to accelerate the depletion of food. This increases the metabolic waste products in semen liquid, subsequently reducing the durability and capability of spermatozoa present (Garner and Hafez 2000).

The results in this study were lower than those reported by Hamdan (1999) on PE goats at the same treatment dose of 0, 50, and 100 µg, which were 69.00 ± 7.76, 75.00 ± 2.64, and 73.66 ± 3.51%, respectively, although the normal motility according to Garner and Hafez (2000) is 60–80%. These values are used as the simplest benchmark in assessing the quality of semen (Pamungkas et al. 2008), and Suyadi et al. (2012) affiliates a high motility with a greater occurrence of fertilization.

The average spermatozoa viability ranges from 57% to 63%, with the specific values at T0, T1, and T2 being 63.00 ± 0.14, 59.00 ± 0.12, and 57.00 ± 0.14% ($P > 0.05$), respectively. Furthermore, the result for Nubian goats in this study was relatively lower compared with the PE species as reported by Hamdan (1999), designating 81.33 ± 3.05, 85.67 ± 1.53, and 86.33 ± 0.58%, at the similar treatment dose. Accord-

ing to Hastono et al. (2013), the live spermatozoa in Anglo-Nubian goats was 58.30 ± 27.30%, with less than 15% sperm (Bintara 2011). Moreover, the occurrence of living and dead forms is often influenced by nutritional and environmental factors. This is in line with the study by Wahyuningsih et al. (2014), who indicated age, genetic, temperature, season, and feed as the factors influencing the quality of fresh semen obtained from superior males. According to Hamdan (1999), the high and low percentage recorded as alive on examination is dependent on the time interval between the ejaculation and completing the object.

Spermatozoa abnormalities in T0, T1, and T2 were 33.00 ± 0.382, 15.00 ± 0.199, and 7.00 ± 0.040% ($P < 0.05$), respectively, characterized by an average decline in those treated with GnRH. The nature of abnormality evaluated in this research include folded tail, broken head, and broken tail. Sajjad et al. (2007) reported the possibility for treating buffaloes to decrease the incidence of spermatozoa abnormalities, directly or indirectly influenced by the increase in the testosterone level (Ronayne et al. 1993; Sajjad et al. 2007). This is in accordance with the results obtained in the current study, with the blood serum testosterone levels T0, T1, and T2 reaching 13.16 ± 9.37, 28.13 ± 1.21, and 33.13 ± 2.30 ng/mL, respectively.

The provision of GnRH in Nubian goats conferred no significant effect ($P > 0.05$) on the semen quality, except for abnormalities, possibly due to variation in species, age, season and nutrition; hence, its effect is categorized as sub-optimal. This is in line with Pamungkas et al. (2008), who recorded the influence of the collecting method and frequency, as well as the age, besides by the diversity in species. According to Monaco et al. (2015), a GnRH injection does not significantly affect volume, motility, and viability, although an increase in sperm concentration was expressed in treated camels.

In this research, it was observed that GnRH possesses the capacity to enhance testosterone concentration, and the statistical analysis result on the effect of treatment in all groups after 60 minutes showed significant differences ($P < 0.05$). Furthermore, the groups administered with 50 µg and 100 µg had relatively higher values compared with the control. This is associated with the high level of GnRH in the plasma and is possibly due to the capacity of hormones to increase its content released by the hypothalamus, which subsequently influences the pituitary to release *gonadotropin hormone* (GtH) (Dewantoro et al. 2017).

Sanford et al. (1977) reported the increase of testosterone concentrations in sheep aged 2–3 years after injecting 50 *µg* GnRH compared with controls. This occurs for 3–4 hours after administration, with the peak value recorded at 1 hour after administration, with 4.08 ± 0.54 and 7.97 ± 1.25 ng/mL, respectively. Ronayne et al. (1993) observed that the administration of 250 *µg* GnRH for cows in India increased testosterone levels 10–15 times compared with the controls at 60 minutes post-administration. Hamdan (1999) also reported elevation of the testosterone level in PE goats injected with 50 *µg* and 100 *µg* GnRH (8.28 ± 0.99 and 9.84 ± 1.97 ng/mL, respectively), using blood samples that were collected after 60 minutes. Moreover, Elkhawagah et al. (2011) reported an upsurge in the blood serum testosterone concentrations of 1.73 ± 0.57, 4.61 ± 1.28, and 4.79 ± 1.21 ng/mL on buffaloes aged 15–18 months, using three different GnRH doses of 8, 12, and 16 µg, respectively. In contratry, Kumar et al. (2016) reported the inability of a 10 µg GnRH injection in bulls aged 6–16 months to increase overall testosterone concentration, although this value increased in cows aged 14–16 months (from 0.97 ± 0.08 to 11.4 ± 2.22 ng/mL) after two hours of administration. This was due to the fact that cows under 14 weeks of age are not yet undergoing puberty, resulting from the imperfect hormones present in the body.

Based on aforementioned facts, the highest concentration of the hormone testosterone was generally observed at 60 minutes or 120 minutes after administering GnRH. Also, an increased blood serum level is affiliated with the elevated amount of LH, resulting from the stimulation of the anterior pituitary. This upsurge subsequently promotes the production of testosterone through interstitial cells (McLachlan et al. 1995).

The results show the tendency for an increase in the dose of GnRH to elevate testosterone concentration. This is evidenced by the values recorded in groups of 50 µg and 100 µg, being 28.13 ± 1.2 and 33.13 ± 2.30 ng/mL, respectively. Dewantoro et al. (2017) have stated that the hormone mechanism is highly dose-dependent, as it works normally (optimal) at a certain level; otherwise, the biological potential toward its target is diminished. Meanwhile, there is a possibility that the injected treatment is unable to efficiently stimulate the target release at lower (suboptimal) doses.

The testosterone concentration in Nubian goats evaluated in the control group was 13.16 ± 9.37 ng/mL, indicating a higher value, compared with the Anglo-Nubian species (5.4 ng/mL), as reported by Souza et al. (2011). Also, the record on other types of goats, including the two-year-old white variety, showed a concentration of 4.30 ± 0.47 ng/mL (Polat et al. 2011), while PE showed 6.82 ± 4.18 ng/mL, Kejobong showed 12.00 ± 6.56 ng/mL, and Bligon showed 9.23 ± 4.73 ng/mL (Rachmawati et al. 2014). In addition, the differences were assumed to have occurred as a result of genetic and breed factors.

The mechanism of testosterone elevation due to the higher dose of a GnRH injection is associated with its ability to stimulate the pituitary neurons to release gonadotropin hormone (GtH-I and GtH-II). These are subsequently released systemically, causing an increase in the serum concentration of both hormones (Kusuma et al. 2012), known to play a role

Table 1. Treatment patterns in experimental animals

Subject	Period		
	I	II	III
1	A	B	C
2	B	C	A
3	C	A	B

A – physiological NaCl (control, T0);
B – 50 µg gonadorelin (T1); C – 100 µg gonadorelin (T2)

Table 2. The average (± SD) volume, concentration, motility, viability, abnormality of spermatozoa, and testosterone levels of Nubian goats with GnRH treatment

Treatment	Group		
	Control (T0)	50 **µg** (T1)	100 **µg** (T2)
Volume (mL)	1.8 ± 0.52 [a]	1.5 ± 0.70 [a]	2.6 ± 1.63 [a]
Concentration (10^6/mL)	807 ± 409.98 [a]	895 ± 509.73 [a]	1,215 ± 270.14 [a]
Motility (%)	37.00 ± 0.33 [a]	34.00 ± 0.30 [a]	65.00 ± 0.11 [a]
Viability (%)	63.00 ± 0.14 [a]	59.00 ± 0.12 [a]	57.00 ± 0.14 [a]
Abnormality (%)	33.00 ± 0.38[a]	15.00 ± 0.19 [b]	7.00 ± 0.040 [b]
Testosterone (ng/mL)	13.16 ± 9.37[a]	28.13 ± 1.21[b]	33.13 ± 2.30[b]

[ab]Different superscripts in the same row showed significant difference ($P < 0.05$)

in spermatogenesis. Furthermore, they have been affiliated with the stimulation of gonads in the production of steroid hormones, including testosterone and 17ß-estradiol. Specifically, GtH-I encourages the development and proliferation of Sertoli cells responsible for the production of androgen binding protein (ABP), needed for arousing the spermatogonium to initiate the process of spermatogenesis. In addition, GtH-II stimulates interstitial or Leydig cells to secrete the hormone testosterone, while the interstitial cell stimulating hormone (ICSH) influences the development of seminiferous tubules and Sertoli cells needed in the production of ABP. This conjoins with testosterone to incite spermatogenesis, alongside the collective function of stimulating sperma development.

Conclusion

Based on the result and discussion, it was concluded that GnRH treatments possess the capacity to reduce spermatozoa abnormalities and also increase testosterone concentrations in Nubian goats.

Acknowledgments

The authors are thankful to the laboratory assistant, the veterinarian, and staff of Laboratory of Reproduction and Laboratory of Research, Universitas Syiah Kuala.

Conflict of Interests

The authors declare that they have no conflict of interests.

Author's Contribution

Syafruddin Syafruddin, Faris Iryandi, Riska Asria Sa'adatur Rahmi, Husnurrizal Husnurrizal wrote the manuscript and conducted the research, Hafizuddin Hafizuddin and Tongku Nizwan Siregar conceptualized the research, and Teuku Armansyah TR., Budianto Panjaitan, Arman Sayuti, Amalia Sutriana, Dwinna Aliza revised the final form of the manuscript.

References

1. Akmal, M., Aulanni'am, A., Widodo, M., Sumitro, S., Purnomo, B., Siregar, T., Hambal, M., A, A., S, S., Aliza, D., Sayuti, A., Adam, M., Armansyah, T., Rahmi, E. Inhibin B menurunkan konsentrasi *follicle stimulating hormone* (FSH) pada tikus putih (*Rattus norvegicus*): upaya pengembangan kontrasepsi hormon pria berbasis peptida. J. Kedokt. Hewan. 2015. Vol. 9(1). P. 42-46.
2. Andalusia, R., Mubarak, A.S., Dhamayanti, Y. Respon pemberian ekstrak hipofisa ayam broiler terhadap waktu latensi, keberhasilan pembuahan dan penetasan pada pemijahan ikan komet (*Carassius auratus auratus*). Berk. Ilmi. Perikan. 2008. Vol. 3(1). P. 21-27.
3. Armansyah, T., Barat, E.R.P., Handini, C.V.R., Aliza, D., Sutriana, A., Hamdan, H., Panjaitan, B., Sayuti, A., Siregar, T.N. Concentration and motility of spermatozoa and testosterone level of Kacang goat after seminal vesicle extract administration. Open. Vet. J. 2018. Vol. 8(4). P. 401-410.
4. Ax, R., Dally, M., Didion, B., Lenz, R., Love, C., Varner, D., Hafez, B., Bellin, M. Semen Evaluation, In: Reproduction in Farm Animals. Lippincott Williams & Wilkins, Philadelphia (US). 2000. pp. 363-375.
5. Azawi, O., Al-Khashab, A.N., Al-Kado, N.N. Effect of gonadotropin releasing hormone treatment on semen characteristics and enzymatic activities of Awassi rams in breeding and non breeding seasons. Iran. J. Appl. Anim. Sci. 2012. Vol. 2(1). P. 13-19.
6. Bintara, S. Rasio spermatozoa X:Y dan kualitas sperma pada kambing kacang dan peranakan etawah. Sain. Pet. 2011. Vol. 9(2). P. 65-71.
7. Dewantoro, E., Yudhiswara, N., Farida, D. Pengaruh penyuntikan hormon ovaprim terhadap kinerja pemijahan ikan tengadak (*Barbonymus schwanenfeldii*). J. Ruaya. 2017. Vol. 5(2). P. 1-9.
8. Elkhawagah, A., Kandiel, M., Sosa, G., Abou-El-Roos, M., Abdel-Ghaffar, A., Azab, A. Effect of GnRH analogue on libido and semen characteristics of puberal buffalo bulls. BVMJ. 2011. Vol. 1. P. 28-34.
9. Garner, D., Hafez, E. Spermatozoa and Seminal Plasma, In: Reproduction in Farm Animals. Lippincott Williams & Wilkins, Philadelphia, US. 2000. pp. 96-109.
10. Gholib, G., Wahyuni, S., Kadar, O.H., Adam, M., Lubis, T.M., Azhar, A., Akmal, M., Siregar, T.N., Armansyah, T., Nugraha, T.P. Measurement of serum testosterone in Kacang goat by using enzyme-linked immunosorbent assay (ELISA) technique: The importance of kit validation J. Kedokt. Hewan. 2016. Vol. 10(1). P. 32-36.
11. Hafizuddin, H., Karja, N., Praharani, L., Setiadi, M. Adiponectin and testosterone levels and its correlation with fer-

tility in Anglo-Nubian x Etawah Grade crossbred bucks. Trop. Anim. Sci. J. 2020. Vol. 43(2). P. 110-116.
12. Hamdan, H. Pengaruh pemberian GnRH (*gonadotropin releasing hormone*) terhadap kualitas semen dan kadar testosteron serum kambing peranakan etawah. M.S. thesis, Universitas Gadjah Mada. 1999.
13. Hasbi, H., Gustina, S. Regulasi androgen dalam spermatogenesis untuk meningkatkan fertilitas ternak jantan. Wartazoa. 2018. Vol. 28(1). P. 013-022.
14. Hastono, H., Adiati, U., Praharani, L.Libido, kemampuan kawin dan kualitas sperma kambing dari tiga bangsa. *In*: Prosiding Seminar Nasional Teknologi Peternakan dan Veteriner. 2013. pp. 345-348.
15. Heriyanta, E., Ihsan, M.N., Isnaini, N. Pengaruh umur kambing peranakan etawah (PE) terhadap kualitas semen segar. J. Tern. Tropik. 2014. Vol. 14(2). P. 1-5.
16. Husin, N., Suteky, T., Kususiyah, K. Uji kualitas semen kambing nubian dan peranakannya (kambing nubian x PE) serta kambing boer berdasarkan lama penyimpanan. J. Sain. Pet. Indo. 2007. Vol. 2(2). P. 57-65.
17. Indriani, I., Susilawati, T., Wahyuningsih, S. Daya hidup spermatozoa sapi limousin yang dipreservasi dengan metode *water jacket* dan *free water jacket*. J. Vet. 2013. Vol. 14(3). P. 379-386.
18. Isnaini, N., Wahjuningsih, S. Konsentrasi *gonadotropin releasing hormone* (GnRH) ekstrak otak sapi peranakan friesien holstein betina fase folikuler dan luteal. J. Kedokt. Hewan. 2014. Vol. 8(2). P. 108-110.
19. Kiyma, Z., Adams, T., Hess, B., Riley, M., Murdoch, W., Moss, G. Gonadal function, sexual behavior, feedlot performance, and carcass traits of ram lambs actively immunized against GnRH. J. Anim. Sci. 2000. Vol. 78(9). P. 2237-2243.
20. Klimas, R., Klimiene, A., Pileckas, V. Seasonal effect on sperm morphological properties of European wild boar (Sus scrofa L) in Lithuania. Vet. Med. Zoot. 2012. Vol. 58. P. 20-25.
21. Kumar, B., Pandita, S., Prakash, B., Mallick, S., Mili, B. Responsiveness of prepubertal crossbred bull calves to exogenous GnRH and its impact on reproductive hormones under tropical conditions. SpringerPlus. 2016. Vol. 5. P. 1-5.
22. Kusuma, P., Marhendra, A., Aulanni'am, A., Marsoedi, M. Mechanism of gonadotropin hormone release in catfish (*Clarias* Sp.) upon laserpuncture exposure to reproduction acupoint. Int. J. Basic Appl. Sci. 2012. Vol. 12(06). P. 177-182.
23. Masir, U., Setiadi, M.A., Karja, N.W.K. Status DNA dan karakteristik spermatozoa kauda epididimis domba pascapenyimpanan pada suhu 4°C J. Vet. 2017. Vol. 18(2). P. 167-174.
24. McLachlan, R.I., Wreford, N.G., Robertson, D.M., de Kretser, D.M. Hormonal control of spermatogenesis. Trends. Endrocrinol. Metab. 1995. Vol. 6(3). P. 95-101.
25. Monaco, D., Fatnassi, M., Padalino, B., Aubé, L., Khorchani, T., Hammadi, M., Lacalandra, G.M. Effects of a GnRH administration on testosterone profile, libido and semen parameters of dromedary camel bulls. Res. Vet. Sci. 2015. Vol. 102. P. 212-216.
26. Novita, C.I., Sudono, A., Sutama, I., Toharmat, T. Produktivitas kambing peranakan etawah yang diberi ransum berbasis jerami padi fermentasi. Med. Pet. 2006. Vol. 29(2). P. 96-106.
27. Padrik, P., Hallap, T., Bulitko, T., Januškauskas, A., Kaart, T., Jaakma, Ü. The quality of frozen-thawed semen of young AI bulls and its relation to the grade of Holstein genes and fertility. Vet. Med. Zoot. 2010. Vol. 50(72). P. 59-65.
28. Pamungkas, F., Mahmilia, F., Elieser, S.Perbandingan karakteristik semen kambing boer dengan kacang. *In*: Seminar Nasional Teknologi Peternakan dan Veteriner. 2008. pp. 367-370.
29. Polat, H., Dellal, G., Baritci, I., Pehlivan, E. Annual change of the testosterone hormone in male White goats. Agr. Sci. China. 2011. Vol. 10(2). P. 312-316.
30. Pratomo, H., Yudi, Y. Pemberian pasak bumi memengaruhi kadar testosteron dan spermatozoa kambing peranakan etawah. J. Kedokt. Hewan. 2016. Vol. 10(2). P. 148-152.
31. Rachmawati, L., Ismaya, I., Astuti, P. Level of testosterone, libido, and sperm quality of Bligon, Kejobong, and Etawah crossbred bucks. Anim. Prod. 2014. Vol. 15(2). P. 76-82.
32. Rahmiati, R., Eriani, K., Dasrul, D.Kualitas dan morfologi abnormal spermatozoa sapi aceh pada berbagai frekuensi ejakulasi. *In*: Prosiding Seminar Nasional Biotik 2015. pp. 339-344.
33. Ronayne, E., Enright, W.J., Roche, J.F. Effects of continuous administration of gonadotropin-releasing hormone (GnRH) or a potent GnRH analogue on blood luteinizing hormone and testosterone concentrations in prepubertal bulls. Domest. Anim. Endocrin. 1993. Vol. 10(3). P. 179-189.
34. Sajjad, M., Ali, S., Akhter, S., Ullah, N. Effect of gonadotropin releasing hormone on semen characteristics in Nili-Ravi buffalo bulls. Pak. Vet. J. 2007. Vol. 27(3). P. 153-154.
35. Sanford, L.M., Palmer, W.M., Howland, B.E. Changes in the profiles of serum LH, FSH and testosterone, and in mating performance and ejaculate volume in the ram during the ovine breeding season. J. Anim. Sci. 1977. Vol. 45(6). P. 1382-1391.
36. Situmorang, P. The effects of inclusion of exogenous phospholipid in Tris diluent with different level of egg yolk on the viability of bull spermatozoa. JITV. 2002. Vol. 7(3). P. 181-187.
37. Souza, L., Cruz, J., Neto, M., Nunes, R., Cruz, M. Puberty and sexual maturity in Anglo-Nubian male goats raised in semi-intensive system. Rev. Bras. Zootecn. 2011. Vol. 40(7). P. 1533-1539.
38. Suparman, E., Suparman, E. Peran GnRH agonis. J. Biomed. 2016. Vol. 8(1). P. 1-7.
39. Suyadi, S., Rachmawati, A., Iswanto, N. Effect of α-tocopherol in tris-aminomethane–egg yolk on the semen quality during cold storage in Boer goats. JIIP. 2012. Vol. 22(3). P. 1-8.
40. Tambing, S.N., Toelihiere, M.R., Yusuf, T.L., Purwantara, B., Sutama, K., Situmorang, P.Z. Pengaruh frekuensi ejakulasi terhadap karakteristik semen segar dan kemampuan libido kambing Saanen. J. Sain. Vet. 2003. Vol. 21(2). P. 57-65.
41. Wahyuningsih, A., Saleh, D.M., Sugiyatno, S. Pengaruh umur pejantan dan frekuensi penampungan terhadap volume dan motilitas semen segar sapi Simmental di Balai Inseminasi Buatan Lembang. J. Ilmi. Pet. 2014. Vol. 1(3). P. 947-953.

Received 11 April 2020
Accepted 19 July 2020

Characterization of *Lactococcus Lactis* for Probiotic Properties *in Vitro*

Kristina Kondrotienė, Neringa Kašėtienė
Department of Food Safety and Quality, Veterinary Academy,
Lithuanian University of Health Sciences

Keywords: *antibiotic resistance, hemolytic activity, probiotics, Lactococcus lactis.*

Abstract. *The aim of the study was to investigate some of the probiotic characteristics and safety aspects of selected Lactococcus lactis strains intended to be used in food or feed and isolated from raw cow milk samples. Antibacterial, hemolytic, gelatinase and enzymatic activities, resistance towards seven antibiotic as well as acid and bile salt were examined. In general, all strains were acid and bile salt tolerant, expressed good antibacterial activity against tested food spoilage and pathogenic bacteria such as Listeria monocytogenes, Escherichia coli, Brochotix thermosphacta and others. Tested L. lactis strains expressed acid resistance up to 80%, whereas the highest resistance was observed in strain 8 where after 3 h of incubation at 30°C under an acidic condition the growth of the isolate decreased from 7.47 ± 0.02 to 6.71 ± 0.16* log_{10}*CFU/mL expressing resistance of 90%. All isolates were resistant at 0.3% bile salt with resistance of more than 50%, whereas strains 8 and 25 expressed a growth decrease from 6.81 ± 0.03 to 6.74 ± 0.02* log_{10}*CFU/mL and from 7.06 ± 0.03 to 6.56 ± 0.04* log_{10}*CFU/mL showing resistance of 99% and 93%, respectively. The highest antibacterial activity was expressed by L. lactis strains 24 and 25 against spoilage bacteria Brochothrix thermosphacta. With regard to hemolytic activity, one strain showed α-hemolysis; thus, this strain could not be used as a probiotic culture. Moreover, one strain (L. lactis 25) expressed strong activity of harmful enzyme α-chymotrypsin; thus, this strain also could not be applied as a probiotic strain. Only L. lactis strain 8 exhibited probiotic characteristics in vitro and was evaluated as safe.*

Introduction

According to the definition by the World Health Organization (WHO), probiotics are defined as live microorganisms which, when administered in adequate amounts, provide a health benefit to the host (FAO/WHO, 2001). Some of the potential benefits are maintenance or improvement of the intestinal microbiota, prevention of various gastrointestinal disorders, protection against mucosal infections, and regulation of lactose intolerance (Zhang et al., 2020). Products containing probiotics are beneficial for human nutrition and as animal feed supplements (Duc, Hong, Barbosa, Henriques, Cutting, 2004). For this reason, probiotics have been receiving special attention from farmers that search for alternatives to the use of traditional antibiotics as growth promoters (Sandes et al., 2017; Schofield et al., 2017) and from the food industry for functional food production. The interest is reasonable as numerous conducted studies show that probiotics have increased milk yields and meat production (García-Hernández et al., 2016; Schofield et al., 2017) as well as could have health benefits for humans (Sandes et al., 2017).

In recent years, a tendency of increased use of probiotic bacteria in various food products like cheese and yoghurts has been observed (Kumar and Kumar, 2015). Food products containing probiotics, the so-called functional foods, have several therapeutic benefits like anticancer, hypoglycemic properties, antioxidant, and immunomodulatory effects; therefore, isolation of new probiotic strains with health promoting benefits is of big interest (Abushelaibi, Al-Mahadin, El-Tarabily, Shah, Ayyash, 2017).

The main criteria used for a strain to be used as probiotic is generally recognized as safe (GRAS) status, ability to survive under unfavorable conditions such as low pH and bile salt condition (Zhang et al., 2020), antimicrobial activity against pathogenic bacteria and antibiotic resistance (Abouloifa et al., 2019).

A large group of probiotic microorganisms used in medicine and food production belongs to the lactic acid bacteria (LAB) (Das, Khowala, Biswas, 2016; Han, Kong, Chen, Sun, Zhang, 2017; Kumar, Kumar, 2015). LAB are usually employed in food manufacturing and preservation processes being generally recognized as safe to their host's health (Sandes et al., 2017). Among the LAB group, *Lactococcus lactis* is included in the Qualified Presumption of Safety (QPS) list and authorized for use in the food and feed chain within the European Union (EFSA, 2012). The source of *L. lactis* is diverse and, although *L. lactis* may naturally be found in different environments, it is most widely known for its association with the milk environment (Cavanagh, Fitzgerald, McAuliffe, 2015).

New LAB isolates have to express several properties including tolerance to bile and acid conditions to be considered as probiotic. Moreover, to ensure the safe use of strains as probiotic cultures, it is necessary

Corresponding author: Kristina Kondrotienė, Department of Food Safety and Quality, Veterinary Academy, Lithuanian University of Health Sciences, Tilžės 18, LT-47181, Kaunas, Lithuania. E-mail: kristina.kondrotiene@lsmuni.lt

to evaluate their safety properties like resistance to antibiotic, antibacterial activity, hemolytic and enzymatic activities. Therefore, the aim of the study was to evaluate bile and acid tolerance of potential probiotic *L. lactis* strains, as well as to evaluate their safety aspects.

Materials and Methods

Lactococcus lactis strains

Three tested *Lactococcus lactis* strains were previously isolated and identified from raw cow milk samples (Kondrotiene et al., 2018). Before conducting any experiments, strains were revitalized in MRS broth (Biolife, Milano, Italy) by growing for 18 h at 30°C.

Antibacterial activity of L. lactis strains

Antibacterial activity of *L. lactis* strains was evaluated using an agar spot test (Schillinger, Lücke, 1989). 3 µL of revitalized strains were spotted on the surface of MRS agar (Biolife) and incubated anaerobically in a jar with Anaerogen (Oxoid) for the generation of anaerobic conditions for 24 h at 30°C. Plates were then overlaid with 7 mL soft agar (0.7%) inoculated with 100 µL of the indicator strain and incubated for 24 h at an optimal growth temperature and atmosphere for the indicator strain. All indicator strains used in the study (Table 1) were revitalized before the experiment in the appropriate medium and temperature. Antibacterial activity was evaluated by measuring a clear inhibition zone diameter around the colony of the tested strain.

Antibiotic resistance evaluation

Antibiotic susceptibility was evaluated using MIC Test Strips (Liofilchem) following the manufacturer's instructions. Tested *L. lactis* strains were revitalized on MRS agar plates (Biolife) by growing for 48 h at 37°C. The inoculum suspension of the tested *L. lactis* strains was prepared by selecting a couple of well-isolated *L. lactis* colonies and preparing McFarland 0.5 standard suspension. Each Mueller-Hinton agar (Oxoid, England) plate was streaked with a sterile swab that was previously soaked in the inoculum suspension. Etest strips of tested antibiotics were placed on a dried plate and incubated for 20 h at 37°C. Minimum inhibitory concentrations (MIC) were determined from the MIC reading scale and expressed in µg/mL.

Enzymatic profile evaluation

Enzymatic profiles of three *L. lactis* (8, 24 and 25) strains were assessed using the API ZYM kit (bioMerieux, Marcy-l'Étoile, France). Each well of the API ZYM strip was inoculated with 65 µL of the McFarland 5 standard suspension of overnight cultures of the strains and incubated at 30°C for 4 h. After incubation, ZYM-A and ZYM-B reagents were added to each well and then incubated at 30°C for 5 min. Results were interpreted according to the manufacturer's instructions. Changes of color were scored from 0 to 5. Color reaction grade 0 was interpreted to correspond to a negative reaction, grades 1 and 2 corresponded to a weak reaction, and grades 3, 4, and 5 corresponded to a strong reaction.

Bile and acid tolerance

For evaluation of bile salt tolerance, tested strains were revitalized and 1 mL of culture was transferred into 9 mL of MRS broth containing 0.3% bile salt. Incubation was carried out at 30°C, and the number of viable bacteria counts was determined after 0 h and 24 h incubation on MRS agar plates. Acid tolerance was evaluated using 1 mL of a revitalized strain that was transferred to 9 mL of PBS adjusted to pH 2.5 (with 5M HCl) and incubated at 30°C. The number of viable bacteria counts was evaluated after 0 h and 3 h incubation periods on MRS agar plates (Thirabunyanon, Boonprasom, Niamsup, 2009).

Hemolytic activity

Hemolytic activity was evaluated using plates containing sheep blood agar. After incubation for 48 h at

Table 1. Food spoilage and pathogenic strains used in the study and their revitalization conditions

Strains	Growth Media	Incubation Temperature (°C)	Incubation Conditions
Listeria monocytogenes ATCC 35152	BHI	37	Aerobic
Staphylococcus aureus ATCC 9144	BHI	37	Aerobic
Escherichia coli ATCC 8739	BHI	37	Aerobic
Pseudomonas aeruginosa NCTC 6750	BHI	37	Aerobic
Bacillus cereus ATCC 11778	BHI	30	Aerobic
Salmonella Typhimurium ATCC 13311	BHI	37	Aerobic
Pseudomonas florescens ATCC 13525	BHI	30	Aerobic
Brochotix thermosphacta ATCC 11509	BHI	25	Aerobic

ATCC – American Type Culture Collection; NCTC – National Collection of Type Cultures, a Culture Collection of Public Health England; BHI – Brain Heart Infusion medium.

30°C, hemolytic activity was recorded as β-hemolysis, α-hemolysis and γ-hemolysis (considered as negative hemolysis) represented as clear zones, green zones or halos around the colonies, respectively (Maragkoudakis et al., 2009).

Gelatinase production

Gelatinase production was evaluated using Luria Bertani agar (Liofilchem). Of each revitalized strain, 1 µL was spotted on the surface of LB agar (Liofilchem) supplemented with 3% (w/v) gelatin (Sigma) and incubated at 37°C and 42°C for 48 h, 25°C for 72 h, and 10°C and 15°C for 10 days. After incubation, the plates were kept at 4°C for 4 h, and the hydrolysis of gelatin was indicated by the formation of opaque halos around the colonies (Perin, Miranda, Todorov, Franco, Nero, 2014).

Results

Enzymatic activity evaluation is presented in Table 2. The evaluation revealed that all tested *L. lactis* strains (8, 2 and 25) had strong activities of esterase (C4) and leucine arylamidase. Besides, all tested *L. lactis* strains had weak activity of valine arylamidase. Strain 8 produced high activities of esterase lipase (C8), cystine arylamidase, acid phosphatase and Naphthol-AS-BI-phosphohydrolase. Other *L. lactis* strains had weak or no activities of these enzymes. No or weak activities were determined for alkaline phosphatase, lipase (C14), valine arylamidase, trypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. *L. lactis* strain 25 produced high activity of α-chymotrypsin, while other strains produced no activity of this enzyme.

Table 2 presents results of selected *L. lactis* strains to acid and bile salts. The survival of LAB in low pH of the stomach is important for tolerating the initial acid stress (Kumar, Kumar, 2015). All *L. lactis* strains were resistant to acid pH value 2.5. Strains 8, 24 and 25 expressed acid resistance up to 80%. The highest resistance was observed in strain 8 where after 3 h of incubation at 30°C under the acidic condition the growth of the isolate decreased from 7.47 ± 0.02 to 6.71 ± 0.16 log_{10}CFU/mL expressing resistance of 90%. Strains 24 and 25 expressed a growth decrease from 7.55 ± 0.01 to 6.64 ± 0.04 log_{10}CFU/mL and from 7.96 ± 0.00 to 6.71 ± 0.07 log_{10}CFU/mL showing resistance of 88% and 84%, respectively.

Tolerance to bile salts is an important property for any potential probiotic bacteria and is one of the criteria for a strain to be used as a probiotic culture (Kumar, Kumar, 2015). All isolates were resistant at 0.3% bile salt with resistance more than 50%. Minimum resistance was observed in strain 24 where after 24 h of incubation at 30°C the growth of isolate decreased from 7.15 ± 0.05 to 3.64 ± 0.04 log_{10}CFU/mL expressing resistance of 51%. Strains 8 and 25 expressed a growth decrease from 6.81 ± 0.03 to 6.74 ± 0.02 log_{10}CFU/mL and from 7.06 ± 0.03 to 6.56 ± 0.04

Table 2. Enzymatic activities of *L. lactis* strains evaluated by the API-ZYM test

Enzyme	Strains		
	8	24	25
Alkaline phosphatase	1	0	1
Esterase (C4)	4	3	3
Esterase lipase (C8)	3	2	2
Lipase (C14)	0	0	0
Leucine arylamidase	4	3	4
Valine arylamidase	2	2	2
Cystine arylamidase	3	2	2
Trypsin	0	0	0
α-chymotrypsin	0	0	3
Acid phosphatase	4	1	2
Naphthol-AS-BI-phosphohydrolase	3	2	0
α-galactosidase	0	0	0
β- galactosidase	0	0	0
β- glucuronidase	0	0	0
α-glucosidase	0	0	0
β-glucosidase	0	0	0
N-acetyl-β-glucosaminidase	0	0	0
α-mannosidase	0	0	0
α-fucosidase	0	0	0

Table 3. Tolerance of *L. lactis* strains to acid and bile salt

L. lactis strains	Media			
	MRS	MRS+0.3% bile salt	MRS	PBS pH 2.5
		log_{10}CFU/mL		log_{10}CFU/mL
8	6.81 ± 0.03	6.74 ± 0.02	7.47 ± 0.02	6.71 ± 0.16
24	7.15 ± 0.05	3.64 ± 0.04	7.55 ± 0.01	6.64 ± 0.04
25	7.06 ± 0.03	6.56 ± 0.04	7.96 ± 0.00	6.71 ± 0.07

The presented values are means of three replicates ± standard deviation.
*%: final (CFU/mL)/control (CFU/mL) × 100.

log_{10}CFU/mL showing resistance of 99% and 93%, respectively.

Antibacterial activity evaluation of *L. lactis* strains using the agar spot test method is presented in Table 4 and Fig.1 a. The strains showed an antagonistic capacity against all tested food spoilage and pathogenic bacteria such as *Listeria monocytogenes*, *Escherichia coli*, *Brochotix thermosphacta* and others. All the diameters of the inhibition zones were higher than 10 mm, except for strains 8 and 25. Strain 8 showed the smallest zone of inhibition against *Pseudomonas aeruginosa* and *Bacillus cereus* with diameters of 4 and 7 mm, respectively. Strain 25 showed the smallest zone of inhibition against *Salmonella* Typhimurium and *Pseudomonas florescens* with diameters of 6 and 9 mm, respectively. The highest antibacterial activity was expressed by strains 24 and 25 against spoilage bacteria *Brochothrix thermosphacta* with inhibition zones being 22 and 23 mm, respectively.

Table 5 and Fig. 1 b present results of antibiotic resistance of the tested *L. lactis* strains. None of the tested strains showed resistance to tested antibiotics such as chloramphenicol, clindamycin, streptomycin, gentamicin, tetracycline, erythromycin and ampicillin above the breakpoints provided by the European Food Safety Authority (European Food Safety Authority, 2012).

Table 6 shows the results of hemolytic activity (also see Fig. 1 c) and gelatinase production. In this study, two tested *L. lactis* strains displayed γ-hemolysis, and in contrast one strain (*L. lactis* strain 24) displayed harmful α-hemolysis; therefore, this strain could not be used as a probiotic culture. Phenotypic testing of gelatinase production revealed that none of the tested *L. lactis* strains presented this activity.

Discussion

Regardless of the interest to examine LAB as starter cultures or biopreservatives for their technological properties, there is a growing tendency to evaluate them for probiotic properties (Perin et al., 2014).

Table 4. Antibacterial activity of *Lactococcus lactis* strains

Indicator strains	Source	*L. lactis* strains		
		8	24	25
		The diameters of the inhibition zones around the colonies on agar plate, mm		
Listeria monocytogenes	ATCC 35152	13 ± 1.41	12 ± 0.00	16 ± 1.41
Staphylococcus aureus	ATCC 9144	15 ± 1.41	14 ± 0.00	11 ± 1.41
Escherichia coli	ATCC 8739	12 ± 0.00	11 ± 1.41	12 ± 0.00
Pseudomonas aeruginosa	NCTC 6750	4 ± 0.00	10 ± 0.00	10 ± 0.00
Bacillus cereus	ATCC 11778	7 ± 0.00	10 ± 0.00	12 ± 0.00
Salmonella Typhimurium	ATCC 13311	10 ± 0.00	17 ± 1.41	6 ± 0.00
Pseudomonas fbrescens	ATCC 13525	10 ± 0.00	10 ± 0.00	9 ± 1.41
Brochothrix thermosphacta	ATCC 11509	17 ± 1.41	22 ± 1.41	23 ± 1.41

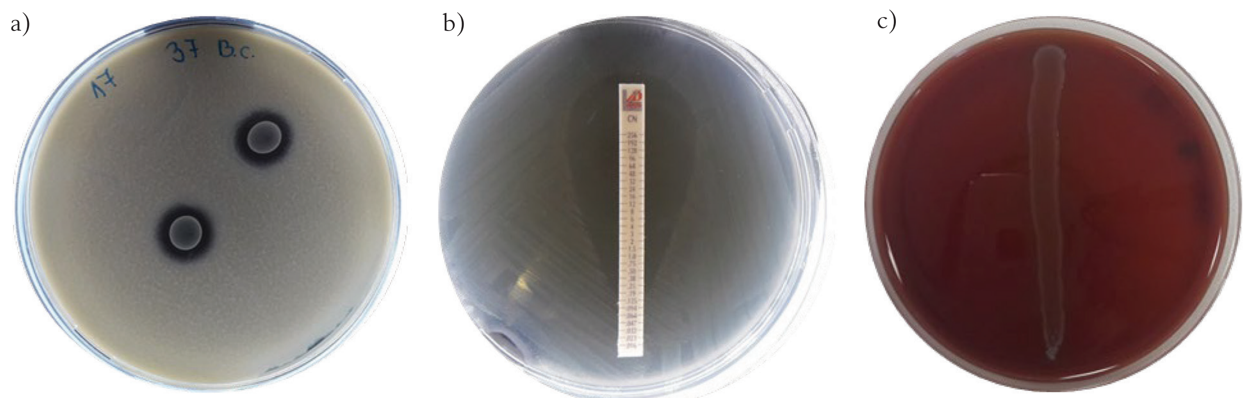

Fig. 1. a) Antibacterial activity evaluation of *L. lactis* strains. Clear zones around the colonies show antibacterial activity against tested microorganisms; b) Antibiotic susceptibility testing of *L. lactis* strains using MIC test strips. Clear zone around the strip show minimum inhibitory concentration; c) Hemolytic activity of *L. lactis* strains (showing γ-hemolysis, which is considered as negative hemolysis).

Table 5. Antibiotic susceptibility of *L. lactis* strains

Antibiotics	Strains			Breakpoints*
	8	24	25	
	Minimum inhibitoryconcentration (MIC), µg/ml			
Chloramphenicol	8	3	3	8
Clindamycin	0.047	0.047	0.064	1
Streptomycin	12	3	0.38	32
Gentamicin	0.25	0.50	0.094	32
Tetracycline	0.19	0.19	0.38	4
Erythromycin	0.094	0.094	0.094	1
Ampicillin	0.094	0.094	0.19	2

*Breakpoints provided by EFSA (2012).

Table 6. Results of hemolytic activity and gelatinase production of *L. lactis* strains

Strains	Hemolytic activity*	Gelatinase production (°C)				
		10	15	25	37	42
8	γ-hemolysis	-	-	-	-	-
24	α-hemolysis	-	-	-	-	-
25	γ-hemolysis	-	-	-	-	-

*α hemolysis means partial hemolysis, γ-hemolysis means absence of hemolysis.
– negative result; + positive result.

The effectiveness of a probiotic strain is species or strain dependent; thus, it is necessary to evaluate each candidate for safety (isolation from suitable habitats, correct identification and antimicrobial susceptibility), functional (resistance to gastrointestinal environment) and beneficial (antagonism against pathogens) properties (FAO/WHO, 2002; García-Hernández et al., 2016). In this study, the probiotic properties of three *L. lactis* strains, previously isolated and identified from raw cow milk samples, were evaluated by *in vitro* tests.

One of the main indicators for a strain to be used as probiotic is its ability to inhibit microbial pathogens (Kumar, Kumar, 2015). The highest antibacterial activity was expressed by two tested *L. lactis* strains 24 and 25 against spoilage bacteria *Brochothrix thermosphacta*. This spoilage organism is associated with spoilage characterized by cheesy, buttery, or sour odors and shares its environmental niche with a member of its sister taxon, *Listeria monocytogenes*, the foodborne pathogen and causative agent of listeriosis (Stanborough, Fegan, Powell, Tamplin, Chandry, 2017). All tested *L. lactis* strains showed good antibacterial activity against this pathogen.

Enzyme production is also one of the main indicators when selecting probiotics (Ji, Jang, Kim, 2015). Strains should not produce harmful enzymes like β-glucosidase, β-glucuronidase (Ji et al., 2015) α-chymotrypsin and N-acetyl-β-glucosaminidase (Abouloifa et al., 2019). Enzymes α-chymotrypsin, N-acetyl-β-glucosaminidase and β-glucuronidase are associated with intestinal diseases (Abouloifa et al., 2019). The possible presence of these enzymes was investigated in this study and strong activity of α-chymotrypsin in *L. lactis* 25 strain was detected; therefore, this strain could not be considered as probiotic. In contrast, β-galactosidase production would be favorable, as this enzyme is considered a beneficial enzyme for a probiotic strain, supporting the reduction of lactose intolerance and milk acidification (Leite et al., 2015); however, none of the tested *L. lactis* strains were producers of this enzyme.

The pH in the human stomach ranges from 1.5 to 4.5, and it has been reported before that acidity has the most negative effect on bacterial growth and viability (Ji et al., 2015). Our study showed that tested *L. lactis* strains 8, 24 and 25 expressed acid resistance up to 80%. These results demonstrate good acid resistance.

Moreover, strains must have good bile tolerance. Physiological concentrations of human bile range from 0.3% to 0.5% (García-Ruiz et al., 2014). It is known that bile salts dissolve membrane lipids leading to the cell's death because of the leakage of the cell contents (Choi, Chang, 2015); therefore, it is important to evaluate the ability of potential probiotic cultures to survive in the presence of bile in order for a probiotic strain to arrive alive in the small intestine or the colon (Kim, Kim, Lee, Kim, Kim, 2012). As it was stated before, all tested *L. lactis* strains were able to resist 0.3% bile salt with resistance above 50%, which reflects a good bile tolerance (Mathara et al., 2008). These results are in accordance with García-Ruiz et

al., 2014 and Kumar & Kumar, 2015. They detected good bile resistance to a variety of LAB strains.

In the antibiotic resistance test, all *L. lactis* strains were susceptible to tested antibiotics. This is a common feature of a probiotic strain (Kumar, Kumar, 2015). However, antibiotic resistance could be considered as an advantage if an antibiotic resistant strain is given during antibiotic treatment. On the other hand, if resistance genes are present on plasmids, they could be transferred to other bacteria including pathogens (Briggiler Marcó, Zacarías, Vinderola, Reinheimer, Quiberoni, 2014).

Hemolytic activity is a typical feature of pathogenic bacteria. This harmful effect may only happen if the ingested bacteria end up in the blood; however, this is an unlikely situation. Nevertheless, this test provides an important information about tested strain's pathogenicity (Miquel et al., 2015). In this study, two tested *L. lactis* strains desplayed γ-hemolysis, and in contrast one strain displayed harmful α-hemolysis; therefore, this strain could not be used as probiotics.

Conclusions

Although strain 24 expressed good antibacterial activity against tested food spoilage and pathogenic bacteria, was acid and bile salt resistant, susceptible to tested antibiotics and was not a producer of harmful enzymes, the strain displayed α-hemolysi; therefore, this strain could not be applied as a probiotic culture. *L. lactis* strain 25 was a producer of α-chymotrypsin; thus, this strain also could not be applied as a probiotic culture.

Only strain 8 exhibited probiotic characteristics *in vitro* and was evaluated as safe.

Results of this study showed that although LAB are generally recognized as safe organisms and are widely used as probiotics, newly isolated strains have to be properly characterized for safety aspects in order to be applied for food or feed production.

More studies are needed to examine probiotic potential of selected a strain and to evaluate its possible beneficial effect and application in food or feed.

References

1. Abouloifa H., Rokni Y., Bellaouchi R., Ghabbour N., Karboune S., Brasca M., Asehraou A. 2019. Characterization of Probiotic Properties of Antifungal *Lactobacillus* Strains Isolated from Traditional Fermenting Green Olives. Probiotics and Antimicrobial Proteins https://doi.org/10.1007/s12602-019-09543-8
2. Abushelaibi A., Al-Mahadin S., El-Tarabily K., Shah N. P., Ayyash M. 2017. Characterization of potential probiotic lactic acid bacteria isolated from camel milk. LWT - Food Science and Technology, *79* 316–325. https://doi.org/10.1016/j.lwt.2017.01.041
3. Briggiler Marcó M., Zacarías M. F., Vinderola G., Reinheimer J. A., Quiberoni A. 2014. Biological and probiotic characterisation of spontaneous phage-resistant mutants of *Lactobacillus plantarum*. International Dairy Journal, *39* (1): 64–70. https://doi.org/10.1016/j.idairyj.2014.05.012
4. Cavanagh D., Fitzgerald G. F., McAuliffe O. 2015. From field to fermentation: The origins of *Lactococcus lactis* and its domestication to the dairy environment. Food Microbiology, *47* 45–61. https://doi.org/10.1016/j.fm.2014.11.001
5. Choi E. A., Chang H. C. 2015. Cholesterol-lowering effects of a putative probiotic strain *Lactobacillus plantarum* EM isolated from kimchi. LWT - Food Science and Technology, *62* (1): 210–217. https://doi.org/10.1016/j.lwt.2015.01.019
6. Das P., Khowala S., Biswas S. 2016. *In vitro* probiotic characterization of *Lactobacillus casei* isolated from marine samples. LWT - Food Science and Technology, *73* 383–390. https://doi.org/10.1016/j.lwt.2016.06.029
7. Duc L. H., Hong H. A., Barbosa T. M., Henriques A. O., Cutting S. M. 2004. Characterization of *Bacillus* Probiotics Available for Human Use. Applied and Environmental Microbiology, *70* (4): 2161–2171. https://doi.org/10.1128/AEM.70.4.2161-2171.2004
8. European Food Safety Authority. 2012. Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance. EFSA Journal, *10* (6): 1–10. https://doi.org/10.2903/j.efsa.2012.2740.
9. FAO/WHO. 2001. Joint FAO/WHO expert consultation on evaluation of health and nutritional properties of prebiotics in food including powder milk with live lactic acid bacteria. *Food and Nutrition Paper* (Vol. 85). https://doi.org/10.1201/9781420009613.ch16
10. FAO/WHO. 2002. Guidelines for the evaluation of probiotics in food. Food and Agriculture Organization of the United Nations and World Health Organization Group Report.(London Ontario, Canada). FAO Food and Nutrition Paper 85
11. García-Hernández Y., Pérez-Sánchez T., Boucourt R., Balcázar J. L., Nicoli J. R., Moreira-Silva J., Halaihel N. 2016. Isolation, characterization and evaluation of probiotic lactic acid bacteria for potential use in animal production. Research in Veterinary Science, *108* 125–132. https://doi.org/10.1016/j.rvsc.2016.08.009
12. García-Ruiz A., González de Llano D., Esteban-Fernández A., Requena T., Bartolomé B., Moreno-Arribas M. V. 2014. Assessment of probiotic properties in lactic acid bacteria isolated from wine. Food Microbiology, *44* 220–225. https://doi.org/10.1016/j.fm.2014.06.015
13. Han Q., Kong B., Chen Q., Sun F., Zhang H. 2017. *In vitro* comparison of probiotic properties of lactic acid bacteria isolated from Harbin dry sausages and selected probiotics. Journal of Functional Foods, *32* 391–400. https://doi.org/10.1016/j.jff.2017.03.020
14. Ji K., Jang N. Y., Kim Y. T. 2015. Isolation of lactic acid bacteria showing antioxidative and probiotic activities from kimchi and infant feces. Journal of Microbiology and Biotechnology, *25* (9): 1568–1577. https://doi.org/10.4014/jmb.1501.01077
15. Kim S. E., Kim Y. H., Lee H., Kim D. O., Kim H. Y. 2012. Probiotic properties of lactic acid bacteria isolated from Mukeunji, a long-term ripened kimchi. Food Science and Biotechnology, *21* (4): 1135–1140. https://doi.org/10.1007/s10068-012-0148-4
16. Kondrotiene K., Kasnauskyte N., Serniene L., Gölz G., Alter T., Kaskoniene V., Malakauskas M. 2018. Characterization and application of newly isolated nisin producing *Lactococcus lactis* strains for control of *Listeria monocytogenes* growth in fresh cheese. LWT - Food Science and Technology 87: 507-514. https://doi.org/10.1016/j.lwt.2017.09.021
17. Kumar A., Kumar D. 2015. Characterization of *Lactobacillus* isolated from dairy samples for probiotic properties. Anaerobe, *33* 117–123. https://doi.org/10.1016/j.anaerobe.2015.03.004
18. Leite A. M. O., Miguel M. A. L., Peixoto R. S., Ruas-Madiedo P., Paschoalin V. M. F., Mayo B., Delgado S. 2015. Probiotic potential of selected lactic acid bacteria strains isolated from Brazilian kefir grains. Journal of Dairy Science, *98* (6): 3622–3632. https://doi.org/10.3168/jds.2014-9265
19. Mathara J. M., Schillinger U., Kutima P. M., Mbugua S. K., Guigas C., Franz C., Holzapfel W. H. 2008. Functional properties of *Lactobacillus plantarum* strains isolated from Maasai

traditional fermented milk products in Kenya. Current Microbiology, *56* (4): 315–321. https://doi.org/10.1007/s00284-007-9084-6
20. Miquel S., Beaumont M., Martín R., Langella P., Braesco V., Thomas M. 2015. A proposed framework for an appropriate evaluation scheme for microorganisms as novel foods with a health claim in Europe. Microbial Cell Factories, *14* 48. https://doi.org/10.1186/s12934-015-0229-1
21. Perin L. M., Miranda R. O., Todorov S. D., Franco B. D. G. de M., Nero L. A. 2014. Virulence, antibiotic resistance and biogenic amines of bacteriocinogenic lactococci and enterococci isolated from goat milk. International Journal of Food Microbiology, *185* 121–126. https://doi.org/10.1016/j.ijfoodmicro.2014.06.001
22. Sandes S., Alvim L., Silva B., Acurcio L., Santos C., Campos M., Nunes Á. 2017. Selection of new lactic acid bacteria strains bearing probiotic features from mucosal microbiota of healthy calves: Looking for immunobiotics through *in vitro* and *in vivo* approaches for immunoprophylaxis applications. Microbiological Research, *200* 1–13. https://doi.org/10.1016/j.micres.2017.03.008
23. Schillinger U., Lücke F. K. 1989. Antibacterial activity of *Lactobacillus sake* isolated from meat. Applied and Environmental Microbiology, *55* (8): 1901–1906. https://doi.org/10.1016/S0168-1605(03)00051-5
24. Schofield B. J., Skarshewski A., Lachner N., Ouwerkerk D., Klieve A. V., Dart P., Hugenholtz P. 2017. Beneficial changes in rumen bacterial community profile in sheep and dairy calves as a result of feeding the probiotic *Bacillus amyloliquefaciens* H57. ARPN Journal of Engineering and Applied Sciences, *12* (10): 3218–3221. https://doi.org/10.1111/ijlh.12426
25. Stanborough T., Fegan N., Powell S. M., Tamplin M., Chandry P. S. 2017. Insight into the genome of *Brochothrix thermosphacta*, a problematic meat spoilage bacterium. Applied and Environmental Microbiology, *83* (5). https://doi.org/10.1128/AEM.02786-16
26. Thirabunyanon M., Boonprasom P., Niamsup P. 2009. Probiotic potential of lactic acid bacteria isolated from fermented dairy milks on antiproliferation of colon cancer cells. Biotechnology Letters, *31* (4): 571–576. https://doi.org/10.1007/s10529-008-9902-3
27. Zhang J., Liu M., Xu J., Qi Y., Zhao N., Fan M. 2020. First insight into the probiotic properties of ten *Streptococcus thermophilus* strains based on *in vitro* conditions. Current Microbiology https://doi.org/10.1007/s00284-019-01840-3.

Received 23 April 2020
Accepted 18 May 2020

Re-Emerging Enzootic Bovine Leukosis in A Cattle Farm in Lithuania: A Case Report

Eglė Rapaliutė[1], Evelina Stončiūtė[1], Marius Masiulis[1,2], Birutė Karvelienė[1], Alvydas Malakauskas[1,2]

[1] *Lithuanian University of Health Sciences, Veterinary Academy, Lithuania*

[2] *State Food and Veterinary Service of the Republic of Lithuania, Emergency Response Department, Lithuania*

Keywords: *Enzootic bovine leukosis, bovine leukemia virus, cattle, risk-based surveillance, disease control.*

Abstract

Enzootic bovine leukosis (EBL) has a severe impact on bovine health and leads to significant economic losses in affected herds. The aim of this case study is to analyze possible sources of repeated occurrence of an EBL infection in a dairy cattle farm in Lithuania. Lithuania is EBL free since 2012; however, increased numbers of disease cases were notified during annual national surveillance in 2017–2019. This case report documents an assessment of a dairy herd with 2 EBL cases within 3 years: EBL was confrmed in 2017 and, after regaining disease free status one year later in 2018, EBL re-entered the same herd in 2019. The most common bovine leukemia virus infection routes were discussed according to the available official data, and iatrogenic transmission was considered as the most plausible reason. There is a constant EBL risk for cattle herds in southwest Lithuania, and this study highlights the need of knowledge about the reasons of confrmed EBL outbreaks to stop the spread of the disease.

Introduction

Enzootic bovine leukosis (EBL) is a disease of cattle caused by bovine leukemia virus (BLV), a member of *Deltaretrovirus* in the family *Retroviridae* (Frie *et al.*, 2015). BLV causes a persistent, life-long infection, induces immune dysregulation with increased susceptibility to other infections and, lastly, may progress to lymphomas in various internal organs (OIE, 2018). Cattle may be infected at any age, including the embryonic stage. Most infections are subclinical, but a proportion of cattle (~30%. Around 60% of infected animals remain asymptomatic and EBL cases in cattle are usually detected during active surveillance of the disease by using serological tests (Kabeya, Ohashi and Onuma, 2001). The modes of transmission of BLV are both vertical and horizontal. The main BLV transmission is horizontal, and any mechanism able to transmit blood or infected lymphocytes between animals can be considered, including the most frequent iatrogenic route with contaminated equipment (EFSA AHAW Panel, 2015) lifelong infection in a subset of B cells. Malignant tumours lymphomas. It has been estimated that movement of infected cattle plays a primary role in the EBL spread between herds, regions or countries (Kobayashi *et al.*, 2014). Vertical transmission via in utero and ingestion of colostrum from BLV-infected cows may account for 10–25% of infections (Mekata *et al.*, 2013). EBL is not a vector-borne disease, but hematophagous insects such as horse flies may contribute to the spread of BLV within a herd by mechanically transferring lymphocytes via biting (Kobayashi *et al.*, 2014).

EBL is a notifiable disease at a national and international level. In the European Union, 22 countries, including Lithuania, have successfully demonstrated freedom from EBL (EC, 2017). After Lithuania officially gained an EBL-free country status in 2012, 20% of bovine herds each year are tested. The annual sampling includes cattle ⩾24 months old, which should be tested at least once every five years. Risk-based sampling for serological EBL surveillance has been suggested in order to increase the sensitivity of surveillance detecting infected herds (Reist, Jemmi and Stärk, 2012). However, EBL still occurs sporadically in Lithuania, but the prevalence of infected herds does not exceed 0.2% annually. There were 21 EBL infected bovine herds in 2017, 27 in 2018, and 21 in 2019. They all were located in south west of Lithuania.

Correspondence to Eglė Rapaliutė, Department of Veterinary Pathobiology, Faculty of Veterinary Medicine, Veterinary Academy, Lithuanian University of Health Sciences, Tilžės 18, LT-47181 Kaunas, Lithuania E-mail: egle.rapaliute@lsmu.lt

Methodology

The data regarding a confirmed EBL outbreak in a dairy cattle farm including herd structure, animal movement, laboratory diagnostics, and control and management strategy were used from the National Veterinary Information Management System and from the EU Animal Disease Notification System (ADNS). Laboratory testing was carried out from cattle blood by performing an enzyme-linked immunosorbent assay (ELISA) test at the National Food and Veterinary Risk Assessment Institute, which is the reference laboratory for animal diseases in Lithuania.

EBL Case presentation

During 2017–2019, 41 of 69 EBL infected herds were found in Marijampolė county in the southwestern part of Lithuania. Since 2017 when risk-based surveillance for EBL was implemented, 100% of bovine herds in affected municipalities of Marijampolė county, including all cattle aged ⩾12 month in a herd, were tested, and increased numbers of BLV-positive animals were confirmed.

The bovine herd taken for this case study was tested for EBL since 2011. The first occurrence of EBL in this herd was in 2017, where 1 animal of 5 tested was confirmed as being positive (Fig. 1)**.** The cow was introduced into the herd in 2008 and since then until 2017 was tested 3 times with negative results. After slaughtering the positive animal, the herd was tested BLV-negative 3 times and regained its disease-free status in April 2018. During the next herd test in February 2019, EBL was confirmed again. The test showed that 1 of 7 tested cows was positive for EBL. A BLV-positive cow was born in the herd in 2008 (Table 1). Between the outbreaks in 2017 and 2019, this cow was tested negative for EBL 3 times. During the period between the last herd test for EBL and the outbreak in 2019, no animals were introduced into the herd. During both EBL outbreaks, positive cows did not show any clinical symptoms of the disease. Both positive animals from the outbreaks in 2017 and 2019 as well as their calves were slaughtered under the supervision of veterinary authorities. All animals in the infected herd that were more than 12 months old were tested serologically at least 2 times within the year. The herd regained its official disease-free status in 2020.

Discussion

According to the scientific literature, bovine movement, iatrogenic transmission, role of hematophagous insects are the most commonly discussed routes of BLV transmission (EFSA AHAW Panel, 2015)lifelong infection in a subset of B cells. Malignant tumours (lymphomas. These possible routes were observed according to the available official data, and the findings suggest the following hypothesis:

1) Hypothesis of EBL spread with the bovine movement

BVL spread between herds is believed to be facilitated by the movement of infected animals (EFSA AHAW Panel, 2015)lifelong infection in a subset of B cells. Malignant tumours (lymphomas. In our case study, after tracing all possible contacts of both in-

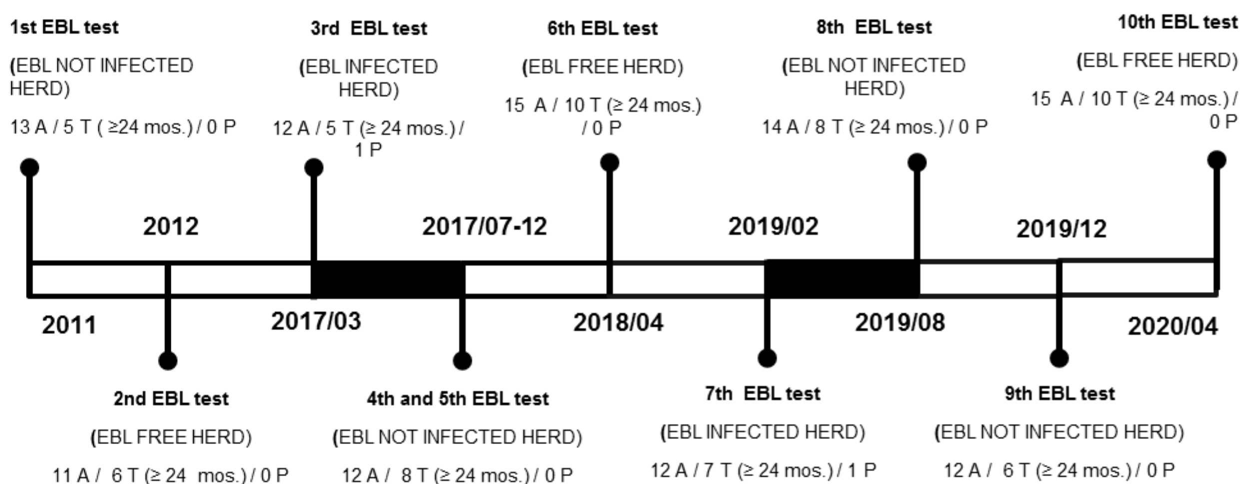

Fig. 1. EBL testing timeline of the outbreak herd.
A – total number of animals, T – tested animals, P – BLV-positive

Table 1. Descriptive information of EBL infected animals in 2017 and 2019 EBL outbreaks in the farm

EBL positive cattle characteristics	Case of 2017	Case of 2019
Age (in years)	8.6	11.2
Movement	1 time	0 times
Born in the herd / moved into the herd	Moved into the herd	Born in the herd
Number of offspring and their EBL status	7 (4 not tested, 3 negative)	7 (3 not tested, 4 negative)
Duration between the last negative EBL test and outbreak	4 years 1 month	9 months

fected cows, no connections between them and other BLV-positive cows from other herds were detected. The owner could not have brought BLV in with infected cows as the purchased cows originated from officially disease-free herds and had several EBL negative tests after movement.

2) Hypothesis via iatrogenic transmission

a. Via diagnostics and treatment. Often the virus is transmitted using blood-contained needles, dehorning instruments or contaminated gloves during rectal palpation (EFSA AHAW Panel, 2015)lifelong infection in a subset of B cells. Malignant tumours (lymphomas. In our case study, there was no accessible information about the treatment or the procedures that were carried out in this farm during the studied period. Considering the fact that the herd is being serviced by a visiting veterinarian who also manages other herds in the same region, iatrogenic transmission between herds through contaminated appliances cannot be excluded.

b. Via artificial insemination. The positive cow from the outbreak in 2017 was artificially inseminated in 2015 only. Since the calf of that cow was tested and was negative for EBL, this infection route for the 2017 EBL-positive cow was not likely. The positive cow from the outbreak in 2019 was artificially inseminated in March 2018. Since then, the cow was tested serologically for EBL being about 2 months pregnant and the result was negative. There is no available information about the calf since it was slaughtered soon after the birth without being tested for EBL due to not relevant age for testing.

Hypothesis of the role of horse flies (Tabanus spp.) in BLV transmission

There is a lack of research regarding the impact of hematophagous insects to BLV transmission within and between cattle herds in natural conditions (Panei *et al.*, 2019)the most common neoplastic disease in cattle. The horn fly, a major hematophagous pest of cattle, is able to transmit different diseases in cattle. However, its implication in BLV transmission under a natural environment is still discussed. The objectives of this work were to determine the presence of BLV in horn flies (by sequencing. According to the recent study of entomologists, *Tabanus spp.* insects are present in Lithuania, but the investigated EBL cases occurred during the time when horse flies are still in the stage of larvae (Turčinavičienė, 2018; Mehlhorn *et al.*, 2010). Nevertheless, we have to point out that the possible infection via a mechanical vector could happen during the time between cattle testing. We also assume that, in the case of *Tabanus spp.* involved in the spreading of EBL as a source of infection, there would possibly be more than one individual EBL case in the herd during an outbreak.

Conclusions

In our case study, no connection was found between the EBL outbreak in 2017 and the outbreak in 2019. We assume that in this particular case vertical transmission, mechanical vectors such as horse flies, animal movement or trade cannot be confirmed as being the causes of EBL cases. The most probable cause of EBL in this bovine herd could be related to iatrogenic transmission. This case report could encourage veterinarians and veterinary epidemiologists to perform a precise outbreak investigation of EBL cases considering all possible infection routes, in order to identify the most plausible source of infection and stop the spread of the disease within the regions at risk and at a national level.

References

1. EC (2017) Bovine and Swine Diseases Situation 2017. 2017.
2. EFSA AHAW Panel (2015) 'Scientific opinion on enzootic bovine leukosis.', EFSA Journal, 13(7), p. 63. doi: 10.2903/j.efsa.2015.
3. Frie, M. C. and Coussens, P. M. (2015) 'Bovine leukemia virus: A major silent threat to proper immune responses in cattle', Veterinary Immunology and Immunopathology, 163(3–4), pp. 103–114. doi: 10.1016/j.vetimm.2014.11.014.
4. Kabeya, H., Ohashi, K. and Onuma, M. (2001) 'Host Immune Responses in the Course of Bovine Leukemia Virus Infection', Journal of Veterinary Medical Science, 63(7), pp. 703–708. doi: 10.1292/jvms.63.703.
5. Kobayashi, S. et al. (2014) 'Analysis of risk factors associated with bovine leukemia virus seropositivity within dairy and beef breeding farms in Japan: A nationwide survey', Research in Veterinary Science. Elsevier Ltd, 96(1), pp. 47–53. doi: 10.1016/j.rvsc.2013.11.014.
6. Mehlhorn, H. and Al-rasheid, K. A. S. (2010) 'Life cycle and attacks of ectoparasites on ruminants during the year in Central Europe : recommendations for treatment with insecticides (e . g ., Butox ®)', pp. 425–431. doi: 10.1007/s00436-010-1957-0.
7. Mekata, H. et al. (2013) 'Paper Evaluation of the natural perinatal transmission of bovine leukaemia virus', pp. 1–5. doi: 10.1136/vr.102464.
8. OIE (2018) Enzootic bovine leukosis (EBL), OIE Terrestrial Manual 2018.
9. Panei, C. J. et al. (2019) 'Study of horn flies as vectors of bovine leukemia virus', Open Veterinary Journal, 9(1), pp. 33–37. doi: 10.4314/ovj.v9i1.6.
10. Reist, M., Jemmi, T. and Stärk, K. D. C. (2012) 'Policy-driven development of cost-effective , risk-based surveillance strategies', Preventive Veterinary Medicine. Elsevier B.V., 105(3), pp. 176–184. doi: 10.1016/j.prevetmed.2011.12.014.
11. Turčinavičienė, J. (2018) Vabzdžių įtaka Afrikinio kiaulių maro plitimui. Vilnius.

Received 15 May 2020
Accepted 19 July 2020

The Fatty Acid Profile of Intramuscular Fat in the *Longissimus Lumborum* Muscle from The Bulls of Black-and-White Holstein-Friesian and Their Cross With the Belgian Blue Breed

Katarzyna Śmiecińska[1], Dorota Kubiak[1]
[1]*Department of Commodity Science and Processing of Animal Raw Materials, Faculty of Animal Bioengineering, University of Warmia and Mazury in Olsztyn, Poland*

Key words: *beef, double-muscled, fatty acids, intramuscular fat.*

Abstract. *The aim of this study was to analyse and compare the fatty acid profle of intramuscular fat (IMF) in the Longissimus lumborum (LL) muscle from the bulls of Black-and-White Holstein-Friesian (HF) and their cross with the Belgian Blue (BB) breed (HF x BB). HF and HF x BB bulls were raised on the same farm, in a tie-stall barn, under identical conditions. The animals were fed farm-made feed. In autumn and winter, they received hay ad libitum, maize silage and ground cereal grain (approx. 2 kg). In summer, they were fed green forage ad libitum, ground cereal grain and hay. In comparison with HF bulls, the IMF in the LL muscle of crossbred HF x BB bulls was characterized by higher concentrations of polyunsaturated fatty acids (PUFAs) and a higher ratio of PUFAs to saturated fatty acids (SFAs) (PUFA/SFA), which indicates that the BB breed with muscular hypertrophy is suitable for commercial crossing. The IMF of crossbred bulls had a higher content of nutritionally important n-3 and n-6 PUFAs, and eicosapentaenoic acid (EPA). The IMF of HF bulls had a higher concentration of conjugated linoleic acid (CLA) with health-promoting properties, but its content in both groups of bulls was comparable with that determined in other cattle breeds.*

Introduction

High-quality beef can be produced by traditional beef cattle breeds and commercial crossbred cattle (Cuvelier et al., 2006). Commercial crossing with beef breeds contributes to progress in beef production in both quantitative and qualitative terms. A beef cattle breed ideally suited for crossing is difficult to find because each breed has its own advantages and disadvantages. One of such breeds is the Belgian Blue (BB), characterized by muscular hypertrophy known as double muscling. The recessive muscular hypertrophy (*mh*) allele that determines double muscling is a mutant form of the myostatin gene (MSTN) (Charlier et al., 1995).

Research shows that the meat of double-muscled cattle and their crosses has a lower percentage of fat (Cuvelier et al., 2006; Moreno et al., 2008), and a higher content of protein (Keady et al., 2013) and unsaturated fatty acids (UFAs) (Aldai et al., 2008; Wiener et al., 2009). In the opinion of consumers from many countries, visible fat in meat is unacceptable and has adverse health effects (Wood et al., 2008). However, fatty acids accumulated in subcutaneous adipose tissue, intermuscular and intramuscular fat (IMF) considerably influence the processing suitability, sensory attributes and nutritional value of meat (Webb and O'Neill, 2008). The concentrations of individual fatty acids in lipids and phospholipids are determined by animal species (Litwińczuk et al., 2012), feed (Scollan et al., 2006; Aldaiet al., 2010), carcass fat content (Wood et al., 2008), the animal's age (Warren et al., 2008), breed and genotype (Ekine-Dzivenuet al., 2014).

Correspondence to Katarzyna Śmiecińska, Department of Commodity Science and Processing of Animal Raw Materials, Faculty of Animal Bioengineering, University of Warmia and Mazury in Olsztyn, Oczapowskiego 5, 10-719 Olsztyn, Poland.
E-mail: katarzyna.smiecinska@uwm.edu.pl

Black-and-White Holstein-Friesians are the most common and the highest-yielding dairy cattle breed in the world. A viable alternative could be crossbreeding between dairy and beef cattle breeds, contributing to the production of high-quality beef. Holstein-Friesians, in particular bulls, have been increasingly used for beef production in recent years. However, it is generally believed that the meat of HF cattle is characterized by lower eating quality than the meat of beef cattle and dairy-beef crosses. Therefore, the HF breed is used mostly for crossing with beef cattle in many countries.

The aim of this study was to analyse and compare the fatty acid profile of IMF in the *Longissimus lumborum* (LL) muscle from the bulls of Black-and-White Holstein-Friesian and their cross with the Belgian Blue breed (HF x BB).

Material and Methods. The experimental materials comprised 10 carcasses of Black-and-White Holstein-Friesian bulls and 10 carcasses of F_1 bulls produced by commercial crossing of HF and Belgian Blue breed (HF x BB). HF x BB bulls were the offspring of double-muscled sires. HF and HF x BB bulls were raised on the same farm, in a tie-stall barn, under identical conditions. The animals were fed farm-made feed. In autumn and winter, they received hay *ad libitum*, maize silage and ground cereal grain (approx. 2 kg). In summer, they were fed green forage *ad libitum*, ground cereal grain and hay.

The bulls were slaughtered in September, at 21 months of age, at a meat processing plant located at a distance of around 90 km from the farm. They were tied during transport. The bulls were weighed on arrival at the meat processing plant. The average body weight of HF and HF x BB was 650 ± 30 kg and 750 ± 33 kg, respectively. Before slaughter, the animals stayed in lairage, in individual pens with free access to water, for around 20 h. They were stunned with the Radical stunning device. The slaughtering and post-slaughter handling were carried out in accordance with the current meat industry regulations (Council Regulation (EC) No 1099/2009 of 24 September 2009 on the protection of animals at the time of killing). After slaughter and post-slaughter processing, the carcasses were weighed and classified in the EUROP system. The pH of the LL muscle was measured approximately 45 min post mortem, between the 1st and 2nd lumbar vertebrae. Weighed carcasses were chilled at a temperature of 1–4°C for 72 h. After chilling, the pH of the LL muscle was measured between the 1st and 2nd lumbar vertebrae. The carcasses were divided into primal cuts. Segments of the LL muscle were collected from the right half-carcasses between the 1st and 2nd lumbar vertebrae. The samples were vacuum-packaged and transported in isothermal containers to the laboratory.

Intramuscular fat was extracted by Soxhlet extraction with diethyl ether as the solvent in the Soxtec™ Avanti 2050 Auto Fat Extraction System (FOSS Analytical, Hilleroed, Denmark) (AOAC 2010). Fatty acid methyl esters were obtained by dissolving the extracted fat in a methanol-chloroform-H_2SO_4 mixture (100:100:1 v/v), followed by methylation according to the Peisker method (Żegarska et al., 1991). Fatty acids were identified by comparing their retention times with those of commercially available reference standards purchased from Supelco, Inc. The percentage share of fatty acids was determined by gas chromatography, using the VARIAN CP-3800 system with a split/splitless injector and a flame-ionization detector (FID). Samples (1 µL) of fatty acid methyl esters were placed on a CP-Sil88 capillary column (length: 50 m, inner diameter: 0.25 mm). Analyses of samples and reference standards were performed under identical conditions, i.e., carrier gas – helium, carrier gas flow rate 1.2 mL/min, injector temperature 225°C, detector temperature 250°C, column temperature 200°C. The fatty acids were divided into the following categories: saturated fatty acids (SFAs), unsaturated fatty acids (UFAs), including monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs), desirable hypocholesterolemic fatty acids (DFAs) (UFAs + C18:0) and undesirable hypercholesterolemic fatty acids (OFAs) (SFAs - C18:0). The following ratios were calculated: DFA/OFA, UFA/SFA, MUFA/SFA, PUFA/SFA, n-6/n-3 PUFA.

The results were processed statistically using STATISTICA software ver. 13.3 (StatSoft, Inc., 2017). Arithmetic means ($\bar{x}$) and standard deviations (s) for all analysed parameters are presented in the Tables. The mean values were compared by the Student's t-test for independent variables. The significance of differences between means was reported at $P \leqslant 0.01$ and $P \leqslant 0.05$.

Results and Discussion

Table 1 presents hot carcass weight, the percentage content of IMF in the LL muscle and the proportions of individual SFAs in the IMF of HF and HF x BB bulls. In the present study, the average hot carcass weight of HF bulls (350 ± 13 kg) was significantly low-

Table 1. The hot carcass weight (kg), the intramuscular fat (IMF) content (%) of the *Longissimus lumborum* muscle and the concentrations of saturated fatty acids (SFAs) in IMF (% of total fatty acids) (means ± SD)

Parameter	HF bulls	HF x BB bulls	Significance
Hot carcass weight	350 ± 13	438 ± 28	**
IMF content of the LL muscle	2.72 ± 1.12	2.01 ± 0.98	NS
C10:0	0.059 ± 0.003	0.057 ± 0.008	NS
C12:0	0.064 ± 0.005	0.085 ± 0.025	*
C14:0	2.968 ± 0.270	2.979 ± 0.216	NS
C15:0	0.399 ± 0.023	0.459 ± 0.115	NS
C16:0	31.102 ± 0.779	31.779 ± 1.229	NS
C17:0	1.066 ± 0.050	1.097 ± 0.139	NS
C18:0	18.374 ± 0.987	16.950 ± 2.195	NS
C20:0	0.141 ± 0.007	0.148 ± 0.039	NS
C22:0	0.073 ± 0.014	0.141 ± 0.034	**
Saturated fatty acids (SFAs)	54.245 ± 0.926	53.695 ± 2.079	NS

NS: non-significant differences ($P > 0.05$).
*Mean values in rows differ at $P \leqslant 0.05$. **Mean values in rows differ at $P \leqslant 0.01$.

er (P ⩽ 0.01) than that of HF x BB bulls (438 ± 28 kg). No significant (P > 0.05) differences in the IMF content of the LL muscle were found between the groups.

Both carcass fat content and the ratio of subcutaneous adipose tissue to IMF and intermuscular fat affect meat quality because the two types of adipose tissue differ in the proportions of lipids and phospholipids, and in the concentrations of individual fatty acids (Wood et al., 2008). The percentage of fatty acids in adipose tissue, regardless of its location, is determined by total body fat percentage (fatness). The percentage of adipose tissue in the carcass increases with age, leading to changes in the proportions of fatty acids. The changes in fatty acid composition are also associated with an increase in carcass lean content in beef cattle (Raes et al., 2004). Dairy cattle have a higher content of IMF and intermuscular fat than beef cattle such as BB since the latter deposit fat mostly within subcutaneous adipose tissue (Wood et al., 2008). According to the literature, the meat of double-muscled cattle (Wiener et al., 2009) and their crosses has considerably lower fat content (Keane and Moloney, 2009; Keane, 2010a, 2010b). In a study by Gotoh et al. (2009), the IMF content of the *Longissimus dorsi* (LD) muscle was only 0.6% in BB cattle, compared with 4.7% in HF cattle.

In the present study, the concentrations of lauric acid (C12:0) (P ⩽ 0.05) and behenic acid (C22:0) (P ⩽ 0.01) in the LL muscle were significantly higher in HF x BB crosses than in HF bulls. The content of the remaining SFAs was similar (P > 0.05) in both groups. Moreover, an analysis of the fatty acid profile of IMF in the LL muscle revealed (P > 0.05) similar percentages of SFAs in both groups of bulls.

In a study by Schiavon et al., (2011), who analysed the *Longissimus thoracis* (LT) muscle of double-muscled Piemontese bulls, the content of C14:0 fatty acid was similar, the content of C16:0 fatty acid was lower, and the content of C18:0 fatty acid was higher than the respective values determined in both groups in our study. Warren et al. (2008) compared the concentrations of SFAs in the *Longissimus* muscle of Aberdeen Angus and HF cattle and found that the content of C14:0, C16:0 and C18:0 fatty acids in IMF was significantly higher in Aberdeen Angus bulls (slaughtered at 24 months of age) than in HF bulls. Sobczuk-Szul et al. (2014) reported no significant differences in the content of C12:0 and C22:0 fatty acids in the LT muscle of HF bulls and HF x Limousin crosses. In the cited study, the concentrations of C14:0, C15:0 and C16:0 fatty acids in IMF were higher in HF x Limousin crosses than in HF bulls. Brugiapaglia et al. (2014) analysed the concentrations of SFAs in the LT muscle of Piemontese, Friesian and Limousin bulls, and found significant (P ⩽ 0.05) differences in the content of C15:0 and C16:0 fatty acids. The effect of breed on the levels of SFAs in the LD muscle of Simmental, Hereford and Charolais cattle was also observed by Ugarković et al. (2013) who reported significant differences in the content of C14:0, C15:0, C16:0 and C17:0 fatty acids among the analysed groups. Horcada et al. (2016) compared the proportions of SFAs in IMF in the LD muscle of young Charolais, Limousin and Retinta cattle and found no significant differences in the content of C12:0 and C14:0 fatty acids, whereas the concentrations of C16:0, C22:0 and C18:0 fatty acids were influenced by breed.

Table 2 presents the percentages of individual UFAs in IMF in the LL muscle of HF and HF x BB

Table 2. The concentrations of unsaturated fatty acids (UFAs) in intramuscular fat (IMF) (% of total fatty acids) in the *Longissimus lumborum* muscle (means ± SD)

Fatty acids	HF bulls	HF x BB bulls	Significance
C14:1	0.586 ± 0.087	0.555 ± 0.169	NS
C16:1	3.459 ± 0.313	4.061 ± 0.919	NS
C17:1	0.884 ± 0.042	1.011 ± 0.124	**
C18:1 cis-9	38.155 ± 0.584	36.559 ± 2.005	**
C20:1	0.078 ± 0.001	0.065 ± 0.009	**
C18:2 n-6	1.881 ± 0.218	3.139 ± 1.228	**
CLA cis-9, trans-11	0.192 ± 0.009	0.159 ± 0.021	**
C18:3 n-3	0.375 ± 0.044	0.429 ± 0.099	NS
C20:2 n-6	0.028 ± 0.004	0.031 ± 0.019	NS
C20:4 n-6	0.065 ± 0.012	0.172 ± 0.107	**
C20:5 n-3 (EPA)	0.054 ± 0.026	0.149 ± 0.102	*
Unsaturated fatty acids (UFAs)	45.755 ± 0.926	46.331 ± 2.085	NS
Monounsaturated fatty acids (MUFAs)	43.160 ± 0.729	42.251 ± 2.788	NS
Polyunsaturated fatty acids (PUFAs)	2.595 ± 0.262	4.079 ± 1.402	**

NS: non-significant differences (P > 0.05).
*Mean values in rows differ at P ⩽ 0.05.**Mean values in rows differ at P ⩽ 0.01.

bulls. IMF in the LL muscle of HF x BB crosses contained significantly ($P \leqslant 0.01$) higher concentrations of margaroleic acid (C17:1), linoleic acid (C18:2 n-6), arachidonic acid (C20:4 n-6) and EPA (C20:5 n-3) ($P \leqslant 0.05$), whereas IMF in the LL muscle of HF bulls had a higher ($P \leqslant 0.01$) content of oleic acid (C18:1 cis-9), gadoleic acid (C20:1) and CLA (C18:2 cis-9, trans-11). In the present study, an analysis of the fatty acid profile of IMF in the LL muscle revealed ($P > 0.05$) similar percentages of UFAs and MUFAs in both groups of bulls, whereas the proportion of PUFAs was significantly ($P \leqslant 0.01$) higher in HF x BB crosses than in HF bulls.

In a study by Litwińczuk et al. (2012), the CLA content of the LL muscle was 0.09% in HF bulls and 0.32% in HF crosses sired by Limousin bulls, and these values are lower and higher, respectively, than those noted in the present experiment. The concentrations of C14:1, C16:1 and C17:1 fatty acids in IMF in the LT muscle were lower in double-muscled Piemontese bulls analysed by Schiavon et al. (2011) than in HF x BB bulls evaluated in this study. Schiavon et al. (2011) reported a higher content of C18:2 fatty acid, and a similar content of CLA, compared with the values noted in HF x BB crosses in the current experiment. In a study by Aldai et al. (2010), the content of CLA (C18:2 cis-9, trans-11) in the LD muscle of double-muscled Asturiana cattle was 0.097 mg/100 g of meat. Heterozygous animals had a higher percentage of CLA in IMF (0.178 mg/100 g of meat), whereas CLA content in cattle with normal muscling was comparable (0.125 mg/100 g of meat) with that noted in double-muscled cattle. Wood et al. (2008) observed a greater increase in carcass fat content with age in beef cattle (Aberdeen Angus) than in dairy cattle (Jersey), accompanied by an increase in the percentage of CLA. Sobczuk-Szul et al. (2014) found no significant differences in the concentrations of C18:2, C22:2 and EPA in IMF in the LD muscle of HF and LM x HF bulls, whereas CLA content was significantly higher in crossbred bulls. Warren et al. (2008) analysed the concentrations of fatty acids in the *Longissimus* muscle of Aberdeen Angus and HF cattle and found that breed had no significant effect on the content of C18:2 n-6 fatty acid. Brugiapaglia et al. (2014) reported a higher percentage of CLA in IMF in the LT muscle of Friesian bulls, compared with Piemontese and Limousin bulls, and no significant differences in EPA content.

The higher content of PUFAs contributed to a more desirable PUFA/SFA ratio ($P \leqslant 0.01$) in the meat of crossbred (HF x BB) bulls (Table 3). In comparison with HF bulls, HF x BB crosses had also significantly higher concentrations of n-3 ($P \leqslant 0.05$) and n-6 ($P \leqslant 0.01$) PUFAs, and a higher ($P \leqslant 0.01$) n-6/n-3 PUFA ratio. No significant ($P > 0.05$) differences in the concentrations of desirable hypocholesterolemic fatty acids (DFAs) and undesirable hypercholesterolemic fatty acids (OFAs) or the DFA/OFA, UFA/SFA and MUFA/SFA ratios were observed between the groups.

Cattle with muscular hypertrophy and low IMF content have higher levels of PUFAs (Aldaiet al., 2008; Wiener et al., 2009; Fiems, 2012), which was also observed in this study in HF x BB bulls. As a result, the PUFA/SFA ratio in double-muscled cattle is also high, ranging from 0.5 to 0.7 (Cuvelier et al., 2006; Scollan et al., 2006; Wiener et al., 2009), compared with approximately 0.25 in animals with normal muscling (Kołczak, 2008).

In our study, the percentage of SFAs was lower and the percentage of UFAs was higher in the LL muscle of HF x BB bulls than in the LT muscle of double-muscled Piemontese bulls evaluated by Schiavon et al. (2011), and in the LT muscle of double-muscled BB bulls analysed by de Smet et al. (2000). In a study by Sobczuk-Szul et al., (2014), the proportions of SFAs (53.110%) and UFAs (46.846%) in

Table 3. The fatty acids groups and ratios in the *Longissimus lumborum* muscle (means ± SD)

Parameter	HF bulls	HF x BB bulls	Significance
Hypocholesterolemic fatty acids DFAs (UFAs + C18:0)	64.129 ± 0.988	63.281 ± 2.099	NS
Hypercholesterolemic fatty acids OFAs (SFAs – C:18:0)	35.871 ± 0.977	36.745 ± 2.455	NS
DFA / OFA ratio	1.788 ± 0.985	1.722 ± 239	NS
UFA / SFA ratio	0.844 ± 0.032	0.863 ± 0.096	NS
MUFA / SFA ratio	0.796 ± 0.027	0.787 ± 0.079	NS
PUFA / SFA ratio	0.048 ± 0.006	0.076 ± 0.019	**
n-3	0.429 ± 0.544	0.578 ± 0.187	*
n-6	1.974 ± 0.845	3.342 ± 1.119	**
n-6 / n-3 PUFA ratio	4.601 ± 0.853	5.782 ± 0.598	**

NS: non-significant differences ($P > 0.05$).
*Mean values in rows differ at $P \leqslant 0.05$.
**Mean values in rows differ at $P \leqslant 0.01$.

the LD muscle of HF bulls were very similar to those noted in the present experiment. In comparison with our findings, Litwińczuk et al. (2012) noted a lower percentage of SFAs (52.24%) and PUFAs (2.62%), and a higher percentage of UFAs (47.75%) and MUFAs (45.135%) in the LT muscle of HF bulls. The cited authors also demonstrated that cattle breed exerted a significant effect on the content of CLA and PUFAs, and the PUFA/SFA ratio in IMF. Aldai et al. (2008) compared the concentrations of n-6 and n-3 PUFAs in the LT muscle of Asturiana bulls with and without the double-muscling phenotype and found that the n-6/n-3 PUFA ratio was significantly higher in double-muscled bulls than in animals with normal muscling. In a study by Sobczuk-Szul et al. (2013), who investigated the fatty acid profile of IMF in the LD muscle of crossbred HF x Limousin bulls, the percentage of DFAs was higher (67.89%) and the percentage of OFAs was lower (32.13%) than the values determined in the current experiment in both groups of bulls.

In the present study, the PUFA/SFA ratio in the LL muscle of HF x BB bulls was similar to that noted by Moreno et al. (2008) in the LD muscle of the same crosses. In our study, the PUFA/SFA ratio was higher and the UFA/SFA and MUFA/SFA ratios were lower than those reported by Sobczuk-Szul et al. (2014) in IMF in the LD muscle of HF bulls and HF x Limousin crosses. Cuvelier et al. (2006) noted a higher PUFA/SFA ratio in IMF in the LT muscle of BB bulls (0.80), compared with Limousin (0.29) and Aberdeen Angus (0.21) bulls. De Smet et al. (2000) demonstrated that a desirable PUFA/SFA ratio and a desirable fatty acid profile of beef were related to a low content of fat in the carcass and in individual muscles. According to the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO), the optimal PUFA/SFA ratio in the human diet is 0.45, and the recommended n-6/n-3 PUFA ratio is 5:1 (Kołczak, 2008). Wijendran and Hayes (2004) estimated the optimal n-6/n-3 PUFA ratio in the diet of healthy adults at around 6:1. The present findings confirm that beef has a highly desirable n-6/n-3 PUFA ratio.

It appears that the observed differences in the fatty acid composition of beef may be due to the progress in analytical methods, effective detection and reliable quantitative analysis of fatty acids, in particular long-chain PUFAs. The different proportions of fatty acids in IMF, reported by various authors, result from the fact that they can be modified by many factors such as animal species, breed, age and diet as well as muscle type. The results of studies conducted by Cuvelier et al. (2006), Wiener et al. (2009) and Aldai et al. (2010) as well as the present findings suggest that double muscling is yet another factor influencing the fatty acid profile of beef.

Conclusions

It can be concluded that, in comparison with HF bulls, the IMF in the LL muscle of crossbred HF x BB bulls was characterized by higher concentrations of PUFAs and a higher PUFA/SFA ratio, which indicates that the BB breed with muscular hypertrophy is suitable for commercial crossing. The IMF of crossbred bulls had a higher content of nutritionally important n-3 and n-6 PUFAs, and EPA. The IMF of HF bulls had a higher concentration of CLA with health-promoting properties, but its content in both groups of bulls was comparable with that determined in other cattle breeds. The use of the BB breed with muscular hypertrophy for commercial crossing may affect the fatty acid profile of IMF in the LL muscle.

References

1. Aldai N., Dugan M.E.R., Nájera A.I.,Osoro K. N-6 and n-3 fatty acids in different beef adipose tissues depending on the presence or absence of the gene responsible for double-muscling. Czech Journal of Animal Science. 2008.T 53(12). P. 515-522.
2. Aldai N., Dugan M.E.R., Juárez M., Martinez A., Osoro K. Double-muscling character influences the trans - 18:1 and conjugated linoleic acid profiles in concentrate-fed yearling bulls. Meat Science. 2010.T 85(1). P. 59-65.https://doi.org/10.1016/j.meatsci.2009.12.004
3. AOAC. 2010. Official Methods of Analysis, 18th ed.; Association of Official Analytical Chemists: Arlington, VA, USA.
4. Brugiapaglia A., Lussiana C.,Destefanis G. Fatty acid profile and cholesterol content of beef at retail of Piemontese, Limousin and Friesian breeds. Meat Science. 2014.T. 96(1). P. 568-573.https://doi.org/10.1016/j.meatsci.2013.08.012
5. Charlier C., Coppieters W., Farnir F., Grobet L., Leroy P.L., Michaux C., Mni M., Schwers A., Vanmanshoven P., Hanset R., Georges M. The mh gene causing double-muscling in cattle maps to bovine Chromosome 2. Mammalian Genome.1995. T. 6. P. 788-792.
6. Council Regulation (EC) No 1099/2009 of 24 September 2009 on the protection of animals at the time of killing. Official Journal of the European Union L 303:1-30.
7. Cuvelier C., Clinquart A., Hocquette J.F., Cabaraux J.F., Dufrasne I., Istasse L.,Hornick J.L. Comparison of composition and quality traits of meat from young finishing bulls from Belgian Blue, Limousin and Aberdeen Angus breeds. Meat Science. 2006.T. 74(3). P. 522-531. https://doi.org/10.1016/j.meatsci.2006.04.032
8. Ekine-Dzivenu C., Chen L., Vinsky M., Aldai N., Dugan M.E.R., McAllister T.A., Wang Z., Okine E., Li C. Estimates of genetic parameters for fatty acids in brisket adipose tissue of Canadian commercial crossbred beef steers. Meat Science. 2014.T. 96(4). P. 1517-1526. https://doi.org/10.1016/j.meatsci.2013.10.011
9. Fiems L.O. Double muscling in cattle: genes, husbandry, carcasses and meat. Animals. 2012.T. 2(3). P. 472-506. https://doi.org/10.3390/ani2030472
10. Gotoh T., Albrecht E., Teuscher F., Kawabata K., Sakashita K., Iwamoto H., Wegner J. Differences in muscle and fat accretion in Japanese Black and European cattle. Meat Science. 2009. T. 82(3). P. 300-308. https://doi.org/10.1016/j.meatsci.2009.01.026
11. Horcada A., Polvillo O., Juárez M., Avilés C., Martínez A.L., Peña F. Influence of feeding system (concentrate and total mixed ration) on fatty acid profiles of beef from three lean cattle breeds. Journal of Food Composition and Analysis. 2016. T. 49. P. 110-116. https://doi.org/10.1016/j.jfca.2016.04.008
12. Keady S.M., Kenny D.A., Ohlendieck K., Doyle S., Keane M.G., Waters S.M. Proteomic profiling of bovine m. longissimus lumborum from crossbred Aberdeen Angus and Belgian Blue sired steers varying in genetic merit for carcass weight.

Journal of Animal Science. 2013. T. 91(2). P. 654-665. https://doi.org/10.2527/jas.2012-5850
13. Keane M.G. Effects of finishing strategy on performance of Belgian Blue x Friesian and Limousin x Friesian steers. Irish Journal of Agricultural and Food Research. 2010a. T. 49(1). P. 27-39.
14. Keane M.G. A comparison of finishing strategies to fixed slaughter weights for Holstein Friesian and Belgian Blue x Holstein Friesian steers. Irish Journal of Agricultural and Food Research. 2010b. T. 49(1). P. 41-54.
15. Keane M.G., Moloney A.P. A comparison of finishing system and duration for spring-born Aberdeen Angus x Holstein-Friesian and Belgian-Blue x Holstein-Friesian steers. Livestock Science. 2009.T. 124(1-3). P. 223-232. https://doi.org/10.1016/j.livsci.2009.02.001
16. Kołczak T. Beef quality. Żywność (Nauka, Technologia, Jakość). 2008. T. 1(56). P. 5-22. (in Polish)
17. Litwińczuk Z., Grodzicki T., Barłowska J., Florek M. Effect of genotype and muscle type on fatty acids profile and cholesterol content in meat of young slaughter cattle. Żywność (Nauka, Technologia, Jakość). 2012. T. 4(83). P. 175-184. (in Polish).
18. Moreno T., Keane M.G., Noci F., Moloney A.P. Fatty acid composition of m. longissimus dorsi from Holstein-Friesian steers of New Zealand and European/American descent and from Belgian Blue x Holstein-Friesian steers, slaughtered at two weights/ages. Meat Science. 2008.T. 78(3). P. 157-169. https://doi.org/10.1016/j.meatsci.2007.05.028
19. Raes K., Haak L., Balcaen A., Claeys E., Demeyer D., Smet S, de. Effect of linseed feeding at similar linoleic acid levels on the fatty acid composition of double-muscled Belgian Blue young bulls. Meat Science. 2004.T. 66(2). P. 307-315.https://doi.org/10.1016/S0309-1740(03)00105-0
20. Schiavon S., Marchi M., de, Tagliapietra L., Bailoni L., Cecchinato A.,Bittante G. Effect of high or low protein ration combined or not with rumen protected conjugated linoleic acid (CLA) on meat CLA content and meat quality traits of double muscled Piemontese bulls. Meat Science. 2011.T. 89. P. 133-142. https://doi.org/10.1016/j.meatsci.2011.03.025
21. Scollan N., Hocquette J.F., Nuernberg K., Dannenberger D., Richardson I., Moloney A. Innovations in beef production systems that enhance the nutritional and health value of beef lipids and their relationship with meat quality. Meat Science. 2006.T. 74(1). P. 17-33. https://doi.org/10.1016/j.meatsci.2006.05.002
22. Smet S., de, Webb E.C., Claeys E., Uytterhaegen L.,Demeyer D.I. Effect of dietary energy and protein levels on fatty acid composition of intermuscular fat in double-muscled Belgian Blue bulls. Meat Science. 2000.T. 56(1). P. 73-79. https://doi.org/10.1016/S0309-1740(00)00023-1
23. Sobczuk-Szul M., Nogalski Z., Wielgosz-Groth Z., Mochol M., Rzemieniewski A., Pogorzelska-Przybyłek P., Purwin C. Fatty acid profile in 4 types of fat depots in Polish Holstein-Friesian and Limousine x Polish Holstein-Friesian bulls. Turkish Journal of Veterinary and Animal Science. 2014.T. 38. P. 189-194. https://doi.org/10.3906/vet-1301-21
24. Sobczuk-Szul M.Wroński M., Wielgosz-Groth Z., Mochol M., Rzemieniewski A., Nogalski Z., Pogorzelska-Przybyłek P.,Purwin C. The effect of slaughter season on the fatty acid profile in four types of fat deposits in crossbred beef bulls. Asian-Australian Journal of Animal Science. 2013.T. 26(2). P. 275-281. https://dx.doi.org/10.5713%2Fajas.2012.12371
25. StatSoft, Inc. Statistica (data analysis software system), version 13.3. StatSoft, Inc. 2017; Tulsa, OK, USA (www.statsoft.com).
26. Ugarković N.K., Ivanković A.,Konjačić M. Effect of breed and age on beef carcass quality, fatness and fatty acid composition. Archiv Tierzucht. 2013.T. 56(97). P. 958-970. https://doi.org/10.7482/0003-9438-56-097
27. Warren H.E., Scollan N.D., Enser M., Hughes S.I., Richardson R.I., Wood J.D. Effects of breed and a concentrate or grass silage diet on beef quality in cattle of 3 ages. I: Animal performance, carcass quality and muscle fatty acid composition. Meat Science. 2008.T. 78(3). P. 256-269. https://doi.org/10.1016/j.meatsci.2007.06.008
28. Webb E.C., O'Neill H.A. The animal fat paradox and meat quality. Review. Meat Science. 2008.T. 80(1). P. 28-36. https://doi.org/10.1016/j.meatsci.2008.05.029
29. Wiener P., Woolliams J.A., Frank-Lawale A., Ryan M., Richardson R.I., Nute G.R., Wood J.D., Homer D., Williams J.L. The effects of a mutation in the myostatin gene on meat and carcass quality. Meat Science. 2009.T. 83(1). P. 127-134. https://doi.org/10.1016/j.meatsci.2009.04.010
30. Wijendran V., Hayes K.C. 2004. Dietary n-6 and n-3 fatty acids balance and cardiovascular health. Annual Review of Nutrition.2004.T. 24. P. 597-615. https://doi.org/10.1146/annurev.nutr.24.012003.132106
31. Wood J.D., Enser M., Fisher A.V., Nute G.R., Sheard P.R., Richardson R.I., Huges S.I., Whittington F.M. Fat deposition, fatty acid composition and meat quality: A review. Meat Science. 2008.T. 78(4). P. 343-358. https://doi.org/10.1016/j.meatsci.2007.07.019
32. Żegarska Z., Jaworski Z., Borejszo Z. Evaluation of the Peisker modified method for extracting methyl esters from fatty acids. Acta Academiae Agriculturae Ac Technicae Olstenensis. Technologia Alimentarum. 1991. T. 24. P. 25-33.

Received 21 May 2020
Accepted 19 June 2020

Conjunctival Microbiota Susceptibility to Antibacterials in Dogs

Dovilė Svetikienė[1], Aidas Grigonis[1], Gintaras Zamokas[1], Birutė Karvelienė[1], Lina Babickaitė[1], Marija Ivaškienė[1], Rita Šiugždinienė[2]

[1] Dr. Leonas Kriaučeliūnas Small Animal Clinic, Veterinary Academy, Lithuanian University of Health Sciences, Lithuania
[2] Institute of Microbiology and Virology, Veterinary Academy, Lithuanian University of Health Sciences, Lithuania

Keywords: *dog, conjuntivitis, susceptibility, antibacterials.*

Abstract. *Bacterial conjunctivitis is the most common ocular pathology in veterinary medicine. The aim of this study was to identify the conjunctival microbiota in dogs with conjunctivitis and to evaluate the antimicrobial susceptibility of the isolated strains. The research was performed at Dr. L. Kriaučeliūnas Small Animal Clinic of Lithuanian University of Health Sciences Veterinary Academy (LUHS VA) in 2018. Samples were tested at the microbiology laboratory of Microbiology and Virology Institute, LUHS VA. Dogs (n = 31) with symptoms of conjunctivitis were selected for primary ophthalmological assessment and research. Samples for bacterial testing were collected from the conjunctival sac. Susceptibility to antibiotics was tested using the Kirby-Bauer disc diffusion method. The investigation revealed that the most frequently isolated bacteria from the conjunctival sac were Gram+ bacteria (89.5%). The identifed bacteria belonged to the families Staphylococcaceae, Corynebacteriaceae, Enterobacteriaceae and Bacillaceae. The most efficient antibacterials against Staphylococcaceae family bacteria were represented by amoxicillin, cephalexin, Corynebacteriaceae by amoxicillin, amoxicillin/clavulanate, Enterobacteriaceae by amoxicillin, amoxicillin/clavulanate, enrofloxacin, gentamicin, tetracycline, trimethoprim/sulfamethoxazole compound, and Bacillaceae by amoxicillin, amoxicillin/clavulanate, cephalexin, cefoxitin, enrofloxacin, erythromycin and gentamicin.*

Introduction

Vision is the most important sense that provides survival superiority for many animals. Eye diseases are common in humans and animals. Therefore, the supply of medications is vast, but only in human medicine. In veterinary medicine, due to the lack of other options, medications designed for human use are the treatment of choice. However, not all human medications are suitable for animals: some of them are really effective, but irritate the eye. In such cases, a human patient simply waits until the irritation fades, whereas an animal immediately starts scratching and rubbing the eye, thus aggravating the inflammation and sometimes spreading it to surrounding tissues.

In a healthy animal, the epithelial barrier, mucin and tears help to rinse the palpebral conjunctiva. Antibacterial components in tears (lysozyme, beta lysine, lactoferrin, blood cells, IgA) protect the eye and its surrounding tissues from bacteria (Nadas et al., 2019).

Bacteria can be cultured from the conjunctival sac of about 70%–90% of normal dogs. The microbiota from the conjunctival sac is represented by mainly Gram+ species, with Staphylococcus spp. (57–70%), Streptococcus spp. (6–43%), Bacillus spp. (6–18%) and Corynebacterium spp. (30–75%) predominating. Predominant Gram– isolates that were recovered from the conjunctival sac in 7%–8% of normal dogs were Pseudomonas spp., Moraxella spp., and small quantities of Klebsiella spp., Neisseria spp. and Fusobacterium spp., and anaerobes were extremely rare. It was found that conjunctival microflora is also related to age, breed, climate, geology and geography and sampling method (Ollivier, 2003, Williams, 2017, Nadas et al., 2019).

Microbiota in the conjunctival sac can be divided into resident and opportunistic pathogenic organisms. Resident bacterial populations are usually isolated from bacteriologic samples of the canine conjunctiva in large numbers. They consist of noninvasive organisms that play an important homeostatic role by competing with pathogenic species for space and nutrients, and also by secretion of active substances that limit their ability to colonize the ocular surface (Williams, 2017).

In animals, conjunctivitis is usually caused by various infectious agents. Sometimes, there are mixed infections caused by several bacterial species or strains. Because non-pathogen bacteria can also be found in the conjunctival sac, the causative agent of inflammation is sometimes difficult to identify. Conjunctivitis is often accompanied by swelling and chemosis, resulting in blinking, sensitivity to light and pain. Animals start rubbing their eyes against surrounding objects, which may lead to traumatic corneal ulcers, and tissues become prone to infection. In dogs with conjunctivitis, the conjunctiva is irritated leading to a decrease in antibacterial enzymes (lactoferrin, lysozyme, and peroxidase). Moreover, the accompanying loss of epithelial integrity, depending on the degree and intensity of the trauma and tear film modifications, favors

Correspondence to Dovilė Svetikienė, Dr. Leonas Kriaučeliūnas Small Animal Clinic, Faculty of Veterinary Medicine, Veterinary Academy, Lithuanian University of Health Sciences, Tilžės 18, LT-47181 Kaunas, Lithuania E-mail: dovile.svetikiene@lsmuni.lt

the colonization of ocular structures by opportunistic bacteria (Pereira et al., 2019).

Often bacterial conjunctivitis for dogs is secondary. The primary cause is usually mechanical irritation from eyelid abnormalities, such as entropion and ectropion, skin wrinkle contact with the cornea of the eyes in dogs of brachycephalic breeds, lash or hair abnormalities, such as ectopic cilia. Distichiasis or trichiasis can also be a cause of the conditions mentioned above. Impaired protective properties of the conjunctiva can lead to infection, and microorganisms of the normal flora can be potential pathogens. Another very probable cause of conjunctivitis is indiscriminate use or long-term application of antimicrobials because it may disrupt the normal conjunctiva microbiota balance and predispose to over-growth of pathogens. Most often, the ongoing infectious process is difficult to control (Athanasiou et al., 2018).

The common type of bacteria cultured from damaged eyes included *Staphylococcus* spp. (40%), *Streptococcus* spp. (64% – β-hemolytic streptococci, and 36% – α-hemolytic streptococci), *P. aeruginosa* (9%), *E. coli* (5%), and *Corynebacterium* spp. (4%). There are *Klebsiella* spp., *Neisseria* spp. and *Moraxella* spp. often found in the samples of the eyes, which are the saprophytic nasopharyngeal microbiota (Haghkhah et al., 2005, Marina et al., 2018, Pereira et al., 2019).

Medications containing gentamicin, chlortetracycline and cloxacillin have been already included in Lithuanian Veterinary Drug Register. Frequently, dog owners practice self-treatment and use eye medications without a veterinarian's prescription. Hence, bacterial strains resistant to antibacterials can develop. In the course of the last 10 years, this has been not only a Lithuanian, but a global problem as well. Although similar research has been performed in Lithuania (Kudirkienė et al., 2006), the number of conjunctivitis cases among dogs is increasing, including the difficult to treat ones. Due to the development of resistant bacteria strains, it is necessary to check the susceptibility of conjunctival isolates to drugs that are used in small animal veterinary medicine in Lithuania.

Many strains of bacteria can be isolated from the conjunctiva. Therefore, a question arises: does the activity spectrum of 3 antibiotic groups ensure the effective treatment of infections caused by Gram+ and Gram– bacteria?

Considering the importance of bacterial conjunctivitis in dogs, the aim of the study was to identify the microorganisms present in dogs with conjunctivitis and evaluate the antimicrobial susceptibility to antibacterials of the identified bacterial species.

Materials and Methods

The research was performed at Dr. L. Kriaučeliūnas Small Animal Clinic of Lithuanian University of Health Sciences Veterinary Academy in 2018. For the study purposes, 31 dogs diagnosed with conjunctivitis were evaluated. Samples for bacteriological testing were collected from the conjunctival sac. Before sampling, a full ophthalmic examination was carried out. The research was performed following the legislation acts of the European Union and the Republic of Lithuania.

Commercial bacteriological swabs were used to sample from the affected eyes into disposable tubes with a transport medium (Transwab, United Kingdom). Samples were tested at a microbiology laboratory of Microbiology and Virology Institute.

The clinically obtained material was disseminated on blood agar (CBA, EO Labs, United Kingdom). Plates with cultures were incubated at 35 ± 2 °**C** temperature for 24 hours in an aerobic and anaerobic environment. If there was no growth, plates were incubated for 48 hours more.

In case it was suspected that the number of bacteria in the sample was too small, the tested material was disseminated into a semi-liquid growth medium (Thioglycollate medium, Oxoid, United Kingdom), kept at +35°C temperature for 24 hours and then reintroduced into Colombia blood agar **(**EO LAB).

In order to identify the genus of staphylococci, the grown cultures were stained by Gram; their growth on Blood and Mannitol salt agar (Mannitol Salt Agar, Liofilchem, Italy) was evaluated. A catalase test was performed. In order to identify *Staphylococcus* species, identifying systems STAPH ID (Microgen, United Kingdom) were used. The results were read using computer program MID Ver1.2. *Enterobacterales* were identified by their colonial morphology, Gram's staining technique, typical growing characteristics on MacConkey agar (Liofilchem, Italy), and SIM medium, where bacteria mobility, hydrogen sulfide, and indole production were evaluated. Preliminary identification of *Corynebacteriaceae* and *Bacillaceae* was performed based on rapid growth on blood agar and morphological properties stained by Gram. *Corynebacteriaceae* - Gram+ rods are seen in pairs in a smear. *Bacillaceae* - Gram+ spore rods.

Susceptibility to antibiotics was determined using the Kirby-Bauer disc diffusion method (EUCAST, 2020). Antibacterial discs were used: amoxicillin (AML; 10 µg), amoxicillin/clavulanate (AMC; 30 µg), cephalexin (CL; 30 µg), cefoxitin (FOX; 30 µg), clindamycin (CD; 2 µg), enrofloxacin (ENR; 5 µg), erythromycin (E; 15 µg), gentamicin (CN; 10 µg), tetracycline (TE; 30 µg), and sulfametoxazole/trimethoprim compound (SXT; 30 µg).

Susceptibility of *Staphylococcus* to amoxicillin and amoxicillin/clavulanic acid compound was evaluated according to susceptibility to cefoxitin and penicillin. The results were evaluated based on EUCAST (European Committee on Antimicrobial Susceptibility Testing) 2020 guidelines for *Enterobacterales, Staphylococcus* spp.

Enterobacterale susceptibility to tetracycline was evaluated based on CLSI M100 standard (CLSI

M100-ED29:2019). Susceptibility to enrofloxacin was evaluated according to CLSI VET08 standard (CLSI VET08 ED4:2018). *Corynebacteriaceae* susceptibility to tetracycline and clindamycin was evaluated according to EUCAST standard (EUCAST, 2020). Susceptibility to other antibiotics was evaluated by marginal values for *Staphylococcus* spp. *Bacillaceae* susceptibility was determined based on susceptiblity/resistance criteria for *Staphylococcus* because there are no established clinical breaking points using the disc method.

Results

Gram+ organisms (89.5%) were more common than Gram– organisms in dogs with conjunctivitis. In a total of 31 samples, there were 57 isolates; the most commonly isolated organisms were *Staphylococcaceae* (64.9%), followed by *Corynebacteriaceae* (15.8%), *Enterobacterales* (10.5%), and *Bacillaceae* (8.8%).

In the samples where bacteria of the family *Staphylococcaceae* were isolated coagulase-negative species were predominant (*Staphylococcus* spp. 73.0%, *Staphylococcus chromogenes* 2.7%), followed by coagulase-positive species (*Staphylococcus aureus* 18.9%, *Staphylococcus intermedius* 5.4%). *Escherichia coli* (33.3%), *Proteus mirabilis* (33.3%), and *Enterobacter* spp. (33.4%) were identified in the samples where bacteria of the family *Enterobacterales* were isolated.

Bacterial associations due to a chronic infectious process were identified in 61.2% of the samples; the most common polymicrobial infection was represented by the association between two different pathogens (41.9%), followed by three pathogens in 16.1%, and four pathogens in 3.2% samples (Table 1). Infections caused by only one pathogen were found in 38.8% of cases.

Amoxicillin and cefalexin showed the greatest efficacy against the *Staphylococcaceae* family bacteria (91.2%), and the least susceptibility was established to amoxicillin/clavulanate (67.6%) (Figure 1). The susceptibility of the identified species of staphylococci to antibacterials differed from the average susceptibility of microorganisms of the *Staphylococcaceae* family (Figure 2). The most efficient antibacterials against *Staphylococcus aureus* were represented by gentamicin, enrofloxacin, clindamycin, and tetracycline (100%), followed by cephalexin, cefoxitin, erythromycin, amoxicillin, and amoxicillin/clavulanate (85.7%). The most efficient antibacterials against *Staphylococcus intermedius* were represented by amoxicillin, amoxicillin/clavulanate, cephalexin, tetracycline, clindamycin, and sulfamethoxazole/trimethoprim (100%), followed by cefoxitin, enrofloxacin, erythromycin, gentamicin, and clindamycin (50%). The most efficient antibacterials against *Staphylococcus chromogenes* were represented by cephalexin, cefoxitin, clindamycin, enrofloxacin, erythromycin, gentamicin, tetracycline and amoxicillin, and sulfamethoxazole/trimethoprim (100%), while and *Staphylococcus chromogenes* was resistant to amoxicillin/clavulanate.

Amoxicillin and amoxicillin/clavulanic acid compound showed the greatest efficacy against the *Corynebacteriaceae* family isolates (100%), and the least susceptibility was established to clindamycin (33.3%).

Amoxicillin, tetracycline, amoxicillin/clavulanate, enrofloxacin, gentamicin, and sulfamethoxazole/trimethoprim showed the greatest efficacy against the *Enterobacterales* isolates (100%), and the least susceptibility was established to cephalexin and cefoxitin (83.3%) (Figure 2). *Proteus mirabilis* isolates were susceptible to all tested antibacterials (100%). Amoxicillin, amoxicillin/clavulanate, tetracycline, gentamicin, and sulfamethoxazole/trimethoprim showed the greatest efficacy against the *E. coli* isolates (100%), followed by cephalexin (50%).

Amoxicillin, amoxicillin/clavulanate, cephalexin, enrofloxacin, gentamicin, and erythromycin showed the greatest efficacy against the *Bacillaceae* family bacteria (100%), and the least susceptibility was established to tetracycline (40%) (Figure 3).

Discussion

The diagnosis and treatment of ocular diseases in dogs are influenced by clinical examination of the eye and the identification of the ocular microbiota. In some cases, empirical treatment can be applied: antibacterial medications are then prescribed suspecting what the causative agent may be and anticipating its susceptibility. Therefore, it is important to know the susceptibility of bacteria that prevail in the region/country, the probability of developing resistance, and its consequences.

The results of our study showed that of all tested samples from the conjunctival sac Gram+ bacteria were

Table 1. Isolates from mixed bacterial infections

Bacteria	Cases	Bacteria	Cases
Staphylococcus spp., *Proteus mirabilis, Corynebacterium* spp.	1	*Staphylococcus* spp., *S. intermedius, Corynebacterium* spp.	1
Staphylococcus spp., *S. aureus*	4	*Staphylococcus* spp., *Proteus mirabilis, Corynebacterium* spp, *S. intermedius*	1
Staphylococcus spp., *S. aureus, S. chromogenes*	1	*Enterobacter* spp., *Bacillus* spp., *Staphylococcus* spp.	1
Staphylococcus spp., *Corynebacterium* spp.	4	*E.coli, Staphylococcus* spp.	1
Staphylococcus spp., *Bacillus* spp.	3		

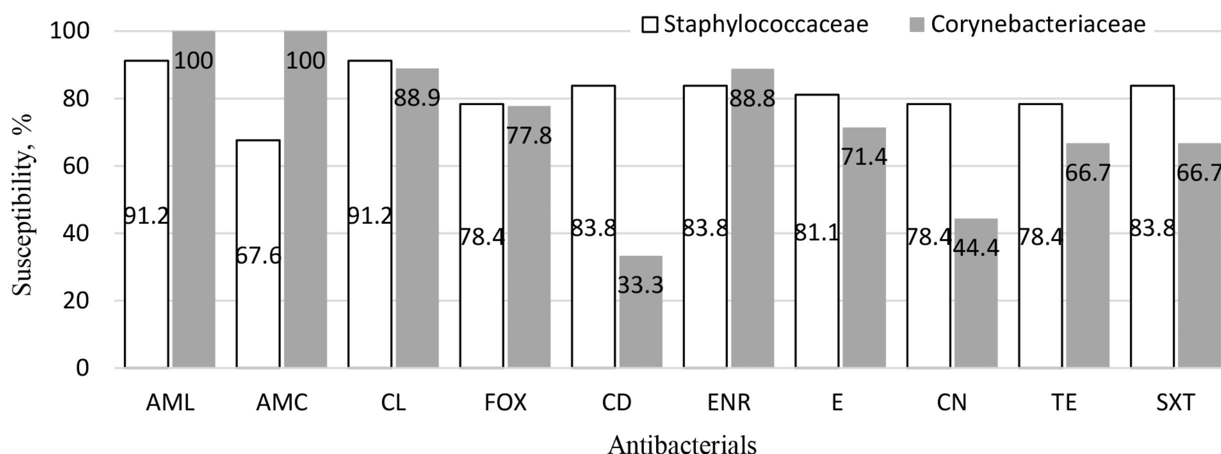

Fig. 1. Susceptibility of *Staphylococcacea* ir *Corynebacteriaceae* family bacterial isolates to antibacterials
AML – amoxicillin, AMC – amoxicillin/clavulanate, CL – cephalexin, FOX – cefoxitin, CD – clindamycin, ENR – enrofloxacin, E – erythromycin, CN – gentamicin, TE – tetracycline, SXT – sulfamethoxazole/trimethoprim.

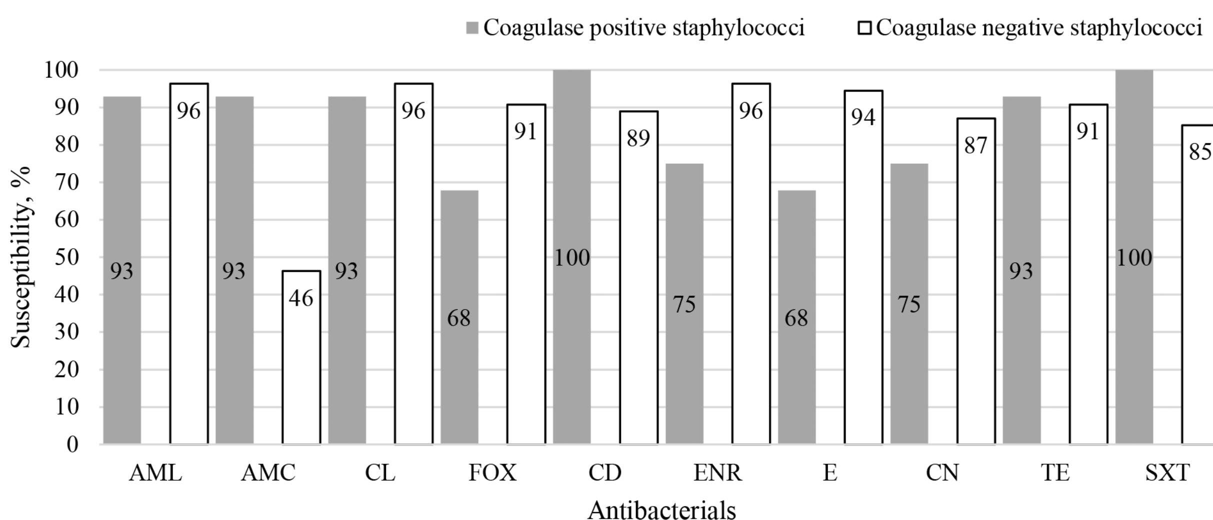

Fig. 2. Susceptibility of the coagulase-positive (*S. aureus* and *S. intermedius*) and coagulase-negative *S. chromogenes* and *Staphylococcus spp.*) staphylococcal isolates to antibacterials (AML – amoxicillin, AMC – amoxicillin/clavulanate, CL – cephalexin, FOX – cefoxitin, CD – clindamycin, ENR – enrofloxacin, E – erythromycin, CN – gentamicin, TE – tetracycline, SXT – sulfamethoxazole/trimethoprim.

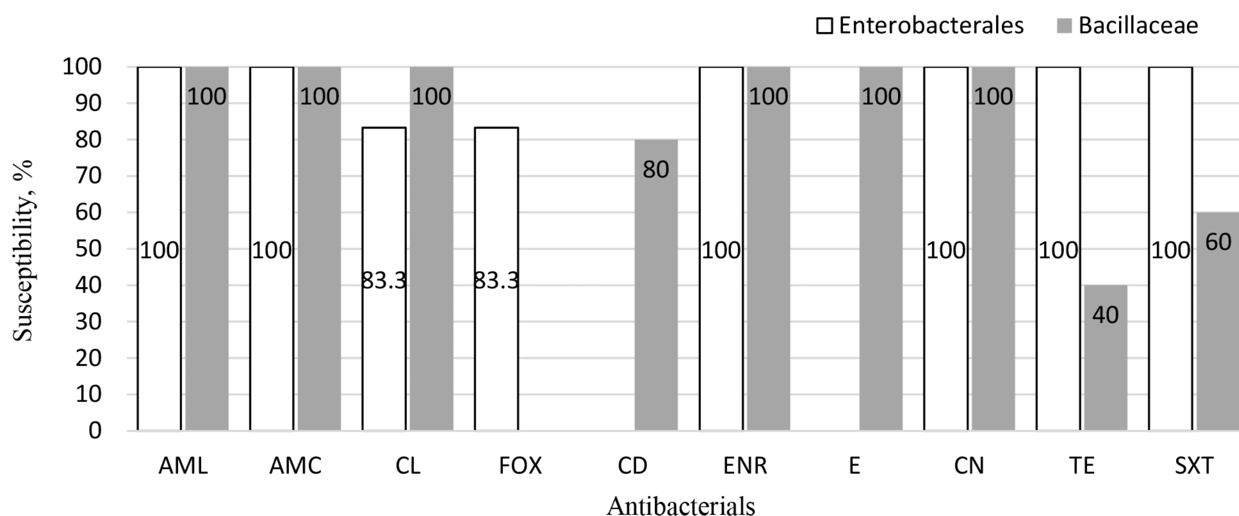

Fig. 3. Susceptibility of *Enterobacterales* and *Bacillaceae* bacterial isolates to antibacterials
AML – amoxicillin, AMC – amoxicillin/clavulanate, CL – cephalexin, FOX – cefoxitin, CD – clindamycin, ENR – enrofloxacin, E – erythromycin, CN – gentamicin, TE – tetracycline, SXT – sulfamethoxazole/trimethoprim.

isolated in 89.5% of cases. Different study reports show a high prevalence of Gram+ bacteria in the conjunctival sac of dogs with conjunctivitis, and it ranges from 56.5% to 67% (Williams, 2017, Pereira et al., 2019). There are some data indicating that up to 44% of conjunctival microbiota consist of Gram– bacteria (Williams, 2017). These results suggest that both Gram+ and Gram– bacterial agents play an important role in the pathogenesis of dogs with conjunctivitis. Previous investigations of the bacterial types associated with conjunctivitis in dogs have shown that Gram+ isolates predominate: *Staphylococcus* spp. (64.8%), *Streptococcus* spp. (18.9%), *Corynebacterium* spp. (0.8–3.5%), and *Bacillaceae* (2.7–4.7%) (Nadas et al., 2019, Pereira et al., 2019). In our research, the most frequent isolates were *Staphylococcus* spp. (64.9%) and *Corynebacterium* spp. (15.8%). However, some authors (Williams, 2017, Das et al., 2019) say that staphylococci are isolated much less frequently, in only about 35.3% of cases. In our research, enterobacteria were isolated in 10.5% of cases, much more often than other authors have reported. The most frequently isolated Gram– bacteria are *Enterobacterales* (2.7–14.4%), as well as *Pseudomonas* spp. (2.7%) (Williams, 2017, Nadas et al., 2019). No *Pseudomonas* spp. were isolated in our research.

Since the eye surface contains not only one single--family of bacteria, infection is also caused by several families of isolates. Our research revealed mixed infections in 61.2% of cases. Research data worldwide show similar results ranging from 29.7% to 53.3%. *Staphylococcus* spp. is a predominant bacterium of this type of infections (Williams, 2017, Nadas et al., 2019). In our research, mixed infections were caused by staphylococci (29.4%), and staphylococci with corynebacterias (29.4%) were most frequently found. Less frequently mixed infections were caused by staphylococci together with *Bacillus* spp. (17.6%).

Amoxicillin/clavulanate totally inhibited the growth of *Corynebacteriaceae*, *Enterobacterales*, and *Bacillaceae* bacterial isolates, and showed a weaker growth-inhibiting effect on *Staphylococcaceae* family isolates (67.6%). Research data by Das et al. (2019) indicate that potentiated amoxicillin had a stronger inhibitory effect on the growth of *Staphylococcus* spp. by 24–26.4%. There are certain data that there is almost no resistance against amoxicillin/clavulanate and exceptions are relatively rare (Pereira et al., 2019).

Our research revealed that non-potentiated amoxicillin acted against conjunctival isolates in 91.2–100% cases. The lowest effect of amoxicillin on the growth of the *Staphylococcaceae* family isolates was revealed. This antibiotic is very popular in clinical practice and, unfortunately, is used not reasonably; hence, there is a possibility of developing bacterial resistance because staphylococci produce beta-lactamases that inhibit the action of antibiotics against beta-lactam bacteria.

Our research showed 83.3–100% of bacteria susceptibility to cephalexin. Cephalexin totally inhibited the growth of the *Bacillaceae* family bacteria, and was slightly weaker against the *Staphylococcaceae* family isolates (91.2%). Meanwhile, research data by Auten (2019) and Das et al. (2019) indicate that cephalexin showed the strongest inhibitory effect on the growth of *Staphylococcaceae* isolates from the dogs' eyes. Data showing lesser activity of cephalexin may possibly be due to frequent use of this antibiotic and developing resistance. The lowest inhibitory effect of cephalexin on the growth of the *Enterobacterales* isolates was found.

Fluoroquinolones can penetrate the cornea and get into hyaloid and eye liquid (Chung et al., 2013). In our research, enrofloxacin inhibited the growth of staphylococci in 83.3% of cases. However, other authors report different data. Das et al. (2019) indicate that susceptibility of staphylococci to fluoroquinolones reaches 100%, while Pereira et al. (2019) report only a 62.5–69.3% effect. Kang et al. (2014) report relative resistance of *Staphylococcus* spp. to fluoroquinolones: only 38.8% of isolates were susceptible (Pereira et al., 2019).

Erythromycin showed a growth-inhibiting action against Gram+ isolates in 71.4–100% of cases. It is one of the safest antibiotics. Other authors report similar data: Awosile et al. (2018) report that susceptibility of staphylococci to erythromycin reaches 62.8–90.7%. Most macrolides show a post-antibiotic effect because they penetrate into phagocytes (neutrophils, macrophages, fibroblasts): their antibacterial activity remains present even when therapeutic concentration decreases (Steel et al., 2012).

Gentamicin was active against isolated bacteria in 44.4–100% of cases. It showed the strongest growth-inhibiting action against *Enterobacterales* and *Bacillaceae* isolates. Its weakest growth-inhibiting action was against the *Corynebacteriaceae* family bacteria. Research of Pereira et al. (2019) reports that aminoglycosides can be used empirically or for preventive treatment of eye infections in dogs. The research revealed that staphylococci were susceptible in 78.4% of cases, slightly more than the research by Williams indicates, i.e., 63% (Williams, 2017). Similar results were obtained by Pereira et al. (2019); meanwhile, Das et al. (2019) indicate more marked susceptibility of staphylococci to gentamicin (85%).

Susceptibility of *Enterobacterales* isolates to gentamicin is not equal. Various studies showed that gentamicin inhibited *E. coli* growth in 100% of cases. Pereira et al. (2019) report it to be 62.5%, whereas *Proteus mirabilis* were susceptible only in 50% of cases (Williams, 2017). Other researchers' data indicate that *E. coli* was resistant in 80% of cases. However, the testing was performed in Taiwan, where gentamicin is the antibiotic of the first choice and is widely used. This might be the reason for such resistance (Williams, 2017), although similar results were obtained by Das et al. (2019). In this case, *E. coli* susceptibility to gentamicin was only 33%, while the growth of *Proteus mirabilis* was completely inhibited by gen-

tamicin. Corynebacteria isolated from the conjunctiva of sick dogs demonstrated lesser susceptibility to gentamicin than the data by Pereira et al. (2019) and Williams (2017) indicate, where gentamicin demonstrated 100% growth-inhibiting activity. Our research showed that gentamicin totally inhibited the growth of *Bacillus* spp. and *Enterobacterales* isolates. This data correspond with the results reported by Williams (2017).

In our research, tetracycline showed a 40–100% inhibiting action against bacteria isolates. Tetracycline showed the most expressed growth-inhibiting effect against *Enterobacterales* isolates; the weakest effect was against *Bacillaceae* family isolates. It was determined that *Staphylococcus intermedius,* one of the most common bacteria found in dogs, is susceptible to tetracycline only in up to 38.6% of cases (Pereira et al., 2019). According to the data from Kang et al. (2014), 94% of *Staphylococcus intermedius* isolated from the conjunctiva of sick dogs were resistant to tetracycline. Our research revealed a comparatively good effect of tetracycline against *S. intermedius* and *S. chromogenes* because the growth of these bacteria were inhibited in 100% of cases. Meanwhile, Pereira et al.'s (2019) data show susceptibility of enterobacteria to tetracycline only in 45.8% cases. Data presented in the literature indicate that *Corynebacterium* spp. is very susceptible to tetracycline, i.e., 100% (Pereira et al., 2019), but our research showed only a 66.7% susceptibility rate.

Sulphametoxazole/trimethoprim compound affected conjunctival isolates in 60–100% of cases. The most expressed growth-inhibiting effect was noticed against *Enterobacterales* isolates; the weakest effect was against *Bacillaceae* family bacteria. Sulfonamides have a wide action spectrum: they act against most Gram+ bacteria. They perfectly penetrate into eye tissues, but many bacteria have become resistant to these synthetic substances; besides, sulfonamides have a very high pH and irritate the conjunctiva (Tačič et al., 2017).

The results of our research revealed that bacteria isolated from the conjunctival sac of dogs diagnosed with conjunctivitis are susceptible to most antibacterials. Based on these results, empirical treatment can be warranted, paying attention to the recommendations regarding choosing first choice and reserved medications. Only unsuccessful treatment would require bacteriological tests that would help to adjust treatment.

Conclusions

1. Gram+ bacteria were most frequently isolated from the conjunctiva of dogs diagnosed with conjunctivitis (89.5%).
2. The best growth-inhibiting effect was demonstrated as follows: against *Staphylococcoceae* family by amoxicillin and cephalexin (91.2%); against *Corynebacteriaceae* by amoxicillin and amoxicillin/clavulanate (100%); against *Enterobacterales* by amoxicillin, amoxicillin/clavulanate, enrofloxacin, gentamicin, tetracycline, and sulphamethoxazole/trimethoprim compound (100%); and against *Bacillaceae* by amoxicillin, amoxicillin/clavulanate, cephalexin, enrofloxacin, erythromycin, and gentamicin (100%).
3. The least susceptible *Staphylococcoceae* isolates were to amoxicillin/clavulanic acid compound (67.4%); *Corynebacteriaceae* to clindamycin (33.3%), *Enterobacterales* to cephalexin and cefoxitin (83.3%); and *Bacillaceae* to tetracycline (40%).
4. Medications included in Lithuanian Veterinary Drugs Registry are sufficient to effectively treat eye diseases in dogs caused by Gram+ and Gram– bacteria.

References

1. Athanasiou, L., Psemmas, D., Papaioannou, N. Conjunctival cytology assessment in dogs and cats. Sampling, diagnostic techniques and findings. Journal of the Hellenic Veterinary Medical Society. 2018. T. 69(1). P. 701-710.
2. Auten C.R., Urbanz J.L., Dees D.D. Comparison of bacterial culture results collected via direct corneal ulcer vs conjunctival fornix sampling in canine eyes with presumed bacterial ulcerative keratitis. Veterinary Ophthalmology. 2019. T. 23. P. 135-140.
3. Awosile B.B., McClure J.T., Saab M.E., Heider L.C. Antimicrobial resistance in bacteria isolated from cats and dogs from the Atlantic Provinces, Canada from 1994-2013. Can Vet J. 2018. T. 59(8). P. 885-893.
4. Chung J.L., Lim E.H., Song S.W., Wroul S.; Yeop K.B., Joon H., Francis M.S., Seo, Yul K. Comparative intraocular penetration of 4 fluoroquinolones after topical instillation. Cornea. 2013. T. 32. P. 1046-1051.
5. Das S., Mishra R., Rath P.K., Mishra B., Mishra C., Behera S.S. Antimicrobial Sensitivity Profile of Eye Infection in Dogs. International Journal of Current Microbiology and Applied Sciences. 2019. T. 8(8). P. 505-511.
6. Haghkhah M., Sarchahi A.A., Molazem M. Conjunctival flora in normal dogs. Juornal of Veterinary Research. 2005. T. 9. P. 79-83.
7. Interneto puslapis. European Commitee on Antimicrobial Susceptibility Testing – (accessed on 2019-08-15). - Available at: https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_10.0_Breakpoint_Tables.pdf.
8. Interneto puslapis. Veterinarinių vaistų registras – (accessed on 2019-12-02). – Available at: http://vetlt1.vet.lt/vr/.
9. Interneto puslapis. Performance Standards for Antimicrobial Susceptibility Testing, 29th - (accessed on 2020-04-25) - Available at: http://em100.edaptivedocs.net/GetDoc.aspx?doc=CLSI%20M100%20ED29:2019&xormat=SPDF&src=BB.
10. Interneto puslapis. Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated From Animals, 4th Edition - (accessed on 2020-04-26) - Available at: http://vet01s.edaptivedocs.info/GetDoc.aspx?doc=CLSI%20VET08%20ED4:2018&scope=user.
11. Interneto puslapis. European Commitee on Antimicrobial Susceptibility Testing – (accessed on 2020-05-21). - Available at: https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Disk_test_documents/2020_manuals/Manual_v_8.0_EUCAST_Disk_Test_2020.pdf.
12. Kang M.H., Chae M.J., Yoon J.W, Lee S.W, Yoo J.H., Can H.M.P. Antibiotic resistance and molecular characterization of ophthalmic Staphylococcus pseudintermedius isolates from dogs. Journal of Veterinary Science. 2014. T. 15, P. 409-415.
13. Kang M.H., Chae M.J., Yoon J.W, Lee S.W, Yoo J.H., Can H.M.P. Resistance to fluoroquinolones and methicillin in ophthalmic isolates of Staphylococcus pseudintermedius from

companion animals. The Canadian veterinary journal. 2014. T. 55(7). P. 678-682.
14. Kudirkienė E., Žilinskas H., Šiugždaitė J. Microbial flora of the dog eyes. Veterinarija ir zootechnika. T. 34 (56). 2006.
15. Marina L., Costa L.O., Costa M.O., Initial description of the core ocular surface microbiome in dogs: Bacterial community diversity and composition in a defined canine population. Veterinary Ophthalmology. 2018. T. 22. P. 337-344.
16. Nadas G.C., Filipoi C.D., Bouari C.M., Buzura-Matei I.A., Chirila F., Novac C.Ş., Fiţ N.I. The Identification and Antimicrobial Susceptibility Profile of Conjunctival Flora from Dogs. Lucrări Ştiinţifice Medicină Veterinară. 2019. T. LII(4). P. 79-82.
17. Olliver F.J. Bacterial Cornea Diseases in Dogs and Cats. Clinical Techniques in Small Animal Practice. 2003. T. 18. P. 193-198.
18. Pereira C.S.G., Zulim F.L.C., Giuffrida R., Cruz A.G, Foglia B.T.D., Benguella H, Batista A.S., Andrade S.F. Antimicrobial susceptibility and minimal inhibitory concentration of bacteria isolated from the eyes of dogs with keratoconjunctivitis sicca. Brazilian Journal of Veterinary Research. 2019. T39(9). P. 757-763.
19. Steel H. C., Theron A. J., Cockeran R., Anderson R., Feldman C. Pathogen-and host-directed anti-inflammatory activities of macrolide antibiotics. Journal of Mediators of Inflammation. 2012. T 2012. Article ID 584262. P. 1-17.
20. Tačič A., Nikolič V., Nikolič L., Savič I. Antimicrobial sulfonamide drugs. Juornal of Advanced technologies. 2017. T 6(1). P. 58-71.
21. Williams D. Bacterial Isolates from Canine External Ocular Disease and their Antimicrobial Sensitivities. ARC Journal of Animal and Veterinary Sciences. 2017. T. 3(3). P. 29-36.

Received 9 March 2020
Accepted 25 May 2020

Impact of a Diet Supplemented with Exoenzymes and Yeast on Predicted Circadian Rhythm of Reticulorumen pH

Ignas Šilinskas[1], Ingrida Monkevičienė[1], Gintarė Dovydaitienė[2], Ilma Tapio[3], Kristina Musayeva[1], Sigitas Japertas[4], Antanas Sederevičius[1] and Rasa Želvytė[1]

[1]*The Research Center of Digestive Physiology and Pathology, Veterinary Academy, Lithuanian University of Health Sciences, Lithuania*

[2]*Departament of Animal Nutrition, Veterinary Academy, Lithuanian University of Health Sciences, Lithuania*

[3]*Natural Resources Institute Finland, Jokioinen, Finland*

[4]*Public Institution Practical training and trial center of Lithuanian University of Health Sciences, Lithuania*

Keywords: *Reticuloruminal pH, circadian rhythm, TMR, cow, exoenzymes, active yeast.*

Abstract. *Reticulorumen pH is affected by rumen fermentation processes and, therefore, is a good indicator of subclinical disease in dairy cattle. The aim of this study was to estimate the effect of exoenzymes and yeast supplementation on the reticulorumen pH circadian rhythm and predict the reticulorumen pH diurnal fluctuation. The study was performed in a commercial dairy farm. Thirty clinically healthy Lithuanian Red cows were assigned into outdoor (OD) and indoor (ID) trials (15 cows each). In each trial, the cows were divided into three homologues groups (5 cows each): control (C) – diet with no supplementation; enzyme (E) – diet supplemented with exoenzymes; enzyme and yeast (EY) – diet supplemented with exoenzymes and active yeast. The reticulorumen pH values were recorded for each cow over a 30-day period using "SmaXtec Premium Bolus" (SmaXtec animal care GmbH. Graz. Austria). The diurnal reticulorumen pH fluctuation was analyzed using the Z-probability equation. In group OD–C, the greatest probability for the reticulorumen pH values to reach maximum was at 06:45 ± 00:20 h. It was by 09 h 14 min (p < 0.001) and 09 h 32 min (p < 0.001) earlier in the day as compared with groups OD–E and OD–EY, respectively. The probability for the reticulorumen pH to reach maximum occurred earlier in group OD–E by 8 h 34 min (p < 0.001) as compared with group ID–E. No significant differences were observed between the groups in the probability for the minimum reticulorumen pH. The time for reticulorumen pH to stay at its maximum value was significantly longer by 06:30 h in trial ID as compared with trial OD. The total amount of time per day of the probability for minimum reticulorumen pH was by 03:34 h (p < 0.05) longer as compared with the probability for maximum reticulorumen pH. According to our results, the Z-probability equation can be used to predict the reticulorumen pH diurnal fluctuation. Total mixed ration supplementation with exoenzymes and active yeast positively affected the time and the length of maximum pH values occurrence.*

Introduction

Rumen plays the most important role of feedstuff digestion in dairy cows. The rumen ecosystem is a sensitive microbiological niche with well-established interactions between different types of microorganisms that are responsible for optimal feed digestion and energy extraction for the host animal (Deng et al., 2008). For efficient forage degradation, not only the quality and the composition of material itself are important, but also physical conditions of the environment where fermentation will be performed. One of the physical parameters is rumen fluid pH. Rumen pH is dependent on rumen microbial fermentation end-products. Optimal rumen pH is close to neutral, pH below 6.0 reduces activity of fiber degrading bacteria, and pH below 5.8 could promote sub-acute ruminal acidosis (SARA), or lead to animal health problems (Kleen at al., 2003; Zebeli et al., 2007; Plaizier et al., 2008). One of the feeding management priorities is to increase fiber digestion and at the same time keep ruminal pH in the normal range (Zebeli et al., 2012). Biological diet supplements, such as active yeast or exoenzymes, can induce non-starch polysaccharide degradation. Exoenzymes are capable of direct hydrolysis of cellulose and hemicellulose matrix, and they also stimulate bacterial attachment to plant cell walls (Adesogan et al., 2019). Active yeast supports anaerobic conditions by reducing the oxygen level in rumen and by increasing ruminal pH through stimulation of lactic acid utilizing bacteria, thus improving conditions for fiber degradation (Fonty and Chaucheyras-Durand, 2006; Adesogan et al., 2019). Both of them affect ruminal pH, but there is a lack of information about an effect of such diet supplements on diurnal pH cycles.

On a commercial dairy farm, rumen pH could be measures by taking ruminal fluid samples using esophagus–rumen probes, or performing rumenocentesis (AlZahal et al. 2007). Both of them are stressful for animals, samples could be contaminated with saliva or blood and have limitations in sampling frequencies. A modern and safe method for animals is to use wireless-indwelling reticulorumen pH measurement systems (Antanaitis et al., 2016). Such systems continuously measure reticulorumen pH and provide large volumes of data, which could be used to calculate average reticulorumen pH at a certain period

Correspondence to Ignas Šilinskas, The Research Center of Digestive Physiology and Pathology, Faculty of Veterinary Medicine, Veterinary Academy, Lithuanian University of Health Sciences, Tilžės 18, LT-47181 Kaunas, Lithuania
E-mail: ignas.silinskas@lsmuni.lt

or amount of time spent below the given threshold. Denwood et al. (2018) suggested that such data could be used to define and diagnose abnormal reticuloruminal pH by identifying deviations from normal pH. A similar study was performed by Josson et al. (2019). They calculated the probability of the reticulorumen pH value to occur below a given threshold in farms with different feeding strategies. But statistical analysis suggested by both studies does not reflect the circadian rhythm of reticulorumen pH.

By predicting the reticulorumen pH diurnal fluctuation, the ruminal digestion and nutrient supply to dairy cows on a given diet can be evaluated. Besides, it is beneficial to avoid ruminal and metabolic diseases, such as acidosis or laminitis. For this reason, AlZahal et al. (2007) used four equations which were growth functions used to describe the growth of animals and plants. But the applied equations only predict reticulorumen pH accumulated time under a given threshold and do not predict the reticulorumen pH diurnal fluctuation. To solve this issue, we considered to use the Z-probability (Z-score) equation (Whitlock, 2005).

We hypothesized that the use of exoenzymes and active yeast supplements with different feeding strategies would affect the reticulorumen pH diurnal fluctuation. The aim of this study was to predict the reticulorumen pH diurnal fluctuation by using Z-probability and evaluate the effect of exoenzymes and yeast supplementation on the reticulorumen pH circadian rhythm.

Materials and Methods

This study was performed on a commercial organic dairy farm (700 cows) in Panevėžys region, Lithuania. Thirty clinically healthy Lithuanian Red cows (an average rectal temperature of 38.7 ± 0.1 °C, rumen motility 5–6 times per 3 min, without signs of mastitis, metritis, or lameness) were used in the experiment. The cows were on average 50 ±7 days in milk and produced on average 31.3 ± 3.0 kg/d of milk. They were assigned to two trials (15 cows each): outdoor (OD), which was performed in the grazing period, and indoor (ID), which was performed in the winter time. During the OD trial, the cows were grazing *ad libitum* on pasture between 11 am and 5 pm, and spent the rest of the time in a free style barn. During the ID trial, the cows were continuously kept in a free stall type barn. In both trials, all cows were offered a total mixed ration (TMR) at 7 am and 5 pm. Diets of both trials were balanced to meet maintenance and production needs (Table 1). The accessibility to water was *ad libitum*. Milking occurred twice daily at 6:00 am and 6:00 pm.

In each trial, the cows were divided into three groups (5 cows each): control (C) – diet with no supplementation; enzyme (E) – diet supplemented with exoenzymes mixture of endo-β-xylanase 37 × 10^4 U/cow/day, endocellulase 45 × 10^4 U/cow/day, and endo-β-glucanase 12 × 10^4 U/cow/day (Vilzim®NSP, UAB Biorro, Lithuania); enzyme and yeast (EY) – diet supplemented with exoenzymes and active dry yeast *Saccharomyces cerevisiae* CNCM-1077, 10 x 10^9

Table 1. The ingredients and chemical composition of farm diet

Item	Value	
	% of diet DM	
	Outdoor trial	Indoor trial
Ingredient		
Grass silage[1]	26	42.8
Corn silage[1]	9	12.3
Straw[1]	3	5.1
High-moisture corn[1]	5	9.1
Ground concentrate mixture[1,2]	29	30.7
Pasture grass[3]	28	0
Diet chemical composition		
DM, %	40.5	44.1
OM, % of DM	71.1	74.6
CP, % of DM	15.08	13.6
NDF, % of DM	43.11	37.1
ADF, % of DM	27.46	22.2

DM – dry matter, OM – organic matter, CP – crude protein, NDF – neutral detergent fiber, ADF – acid detergent fiber.
[1] Ingredients of TMR.
[2] Composition of the concentrate mixture. Dry grains: wheat, triticale, oats; dry ground peas and ground beans, dry rapeseed, soybean meal, sodium bicarbonate, feed chalk, salt, vitamins and trace mineral premix.
[3] Pasture grass: ryegrass, red clover, white clover, alfalfa grass.

CFU/cow/day (Levucell SC, Lallemand Animal Nutrition, France). The corresponding treatment was administered by top-dressing forages and was mixed by using a TMR mixer – feeder "Kuhn Euromix I" (Bucher Industries AG. Switzerland).

The cows were adapted to the diet supplement for 21 days, before each experimental period. The reticulorumen pH values were recorded for 30 days in each trial. All cows were equipped with intrareticuloruminal telemetric sensor device "SmaXtec Premium Bolus" (SmaXtec animal care GmbH. Graz. Austria). The bolus specifications and guidelines for application to animals were described by Antanaitis et al. (2016).

Probabilities for the reticulorumen pH to reach the highest or the lowest values during the diurnal cycle were calculated using the Z-probability table. The Z-probability test takes advantage of the one-to-one mapping of the standard normal curve to the p value of a one-tailed test (Whitlock, 2005). By Z, we mean a standard normal deviation, which would be a daily reticulorumen pH distribution with mean and standard deviation. As Z goes from negative infinity to infinity, P will go from 0 to 1, and any value of P will uniquely be matched with a value of Z and vice versa. The Z values of −1.96 and 1.96 were selected as points in data set to describe 2.5% of the lowest and the highest measurement values in the daily data range, respectively. An equation to find the necessary reticulorumen pH value was used: .

All measured reticulorumen pH values which were equal, above (for maximum) or below (for minimum) the estimated value were selected and marked as −1 or 1, respectively. Time values were also recorded. A probability for pH to reach the maximum or the minimum value at a certain point of time was calculated using the equation:

$$P(A) = \frac{n(E)}{n(S)}$$

where E is positive observations (a sum of −1 or 1) and S is all possible observations (30 days).

A probability in separate trial groups was calculated as means and expressed in percent. For regression analysis, the Pearson correlation coefficient (R) was used. Statistical analysis was performed by ANOVA with the Fisher's least significant difference test. Statistical calculations were carried out using IBM Statistic SPSS version 15 (license No. 9900457; IBM. USA). The results were considered statistically significant when $p \leqslant 0.05$.

Results

In general, the diurnal reticulorumen pH fluctuation of each trial group had two periods when the probability was higher for maximum diurnal pH values and two periods when the probability was higher for the minimum diurnal pH values (Fig. 1). The difference between the treatment groups was in the time when the maximum or the minimum was reached, in the size of probability and the length of periods.

In the first half of the day, the probability for maximum pH values started to increase before the morning feeding. The earliest probability started to occur in group ID–EY at 05:24 ± 00:32 am , and the latest probability occurred in group OD–EY at 06:05 ± 00:41 am . The highest probability was registered in group OD–C (22.67 ± 4.87%) and the lowest probability in group OD–E (11.33 ± 1.8%). The longest period in the first half of the day was recorded in group ID–EY for 05:34 ± 00:21 h, and the shortest was recorded in group OD–E for 01:38 ± 00:17 h. In the first half of the day, the probability for maximum pH values increased fastest in group OD–C (18.46%/h, R = 0.92) and slowest in group ID–EY (4.8%/h, R = 0.67). As group OD–C was compared with group OD–E and group OD–EY, better probability for maximum pH values occurred 09:14 h ($p < 0.001$) and 09:32 h ($p < 0.001$) later in the day, respectively.

After the morning feeding, the probability for maximum pH started to decrease and the probability for minimum pH started to increase. The earliest probability for minimum pH was registered in group OD–E (09:10 ± 00:29 am) and the latest was registered in group OD–EY (11:08 ± 00:34 am). The highest probability was registered in group OD–C (6.67 ± 1.4%) and the lowest probability was in group ID–E (3.33 ± 0.7%). The longest period in the first half of the day of the probability for minimum pH values was recorded in group ID–E for 05:50 ± 00:34 h and the shortest was in group OD–C for 02:16 ± 00:20 h.

In the second half of the day, the reticulorumen pH fluctuation was similar to pH fluctuation in the first half of the day: the probability for maximum pH values had the greatest values within evening feeding and then started to decrease followed by increased probability for minimum pH values. The earliest probability for maximum pH values occurred in group OD–EY at 1:17 ± 00:13 pm and the latest in group ID–E at 3:58 ± 00:47 pm . The probability for maximum pH values ranged from 7.33 ± 1.3% at 3:18 ± 00:38 pm (group OD–C) to 28.89 ± 3.05% at 4:30 pm (± 00:22) (group OD–EY), while the probability increased fastest in group OD–E (10.2%/h, R = 0.87) and slowest in group OD–C (3.67%/h, R = 0.71). The shortest period in the second half of the day of the probability for maximum pH values was recorded in group OD–C for 01:58 ± 00:24 h and the longest was in group ID–EY for 04:50 ± 00:26 h. No significant differences in the length of the periods between treatments were observed.

The probability for minimum reticulorumen pH values was observed during the nighttime in all groups. Group OD–C was the first group when the probability for minimum pH started to increase (at 5:00 ± 00:19 pm). This group had the longest period (10:00 ± 00:20 h) as well. The latest (started to

increase at 8:20 ± 00:17 pm) with the shortest time (08:02 ± 00:14 h) was group ID–EY. The highest probability values of minimum pH was registered in group OD–EY (14.14 ± 2.08%), while the lowest occurred in group ID–E (9.98 ± 0.1%).

The overall probability time length of maximum reticulorumen pH during the day varied from 06:10 h (group OD–E) to 10:20 h (group ID–EY). The probability of minimum reticulorumen pH varied from 8:50 h (group OD–C) to 13:30 h (group OD–E). The probability of minimum reticulorumen pH was by 03:34 h ($p < 0.05$) longer than the probability of maximum reticulorumen pH. As trial ID was compared with trial OD, the period of probability for maximum reticulorumen pH values was longer for trial ID by 06:30 h ($p < 0.05$).

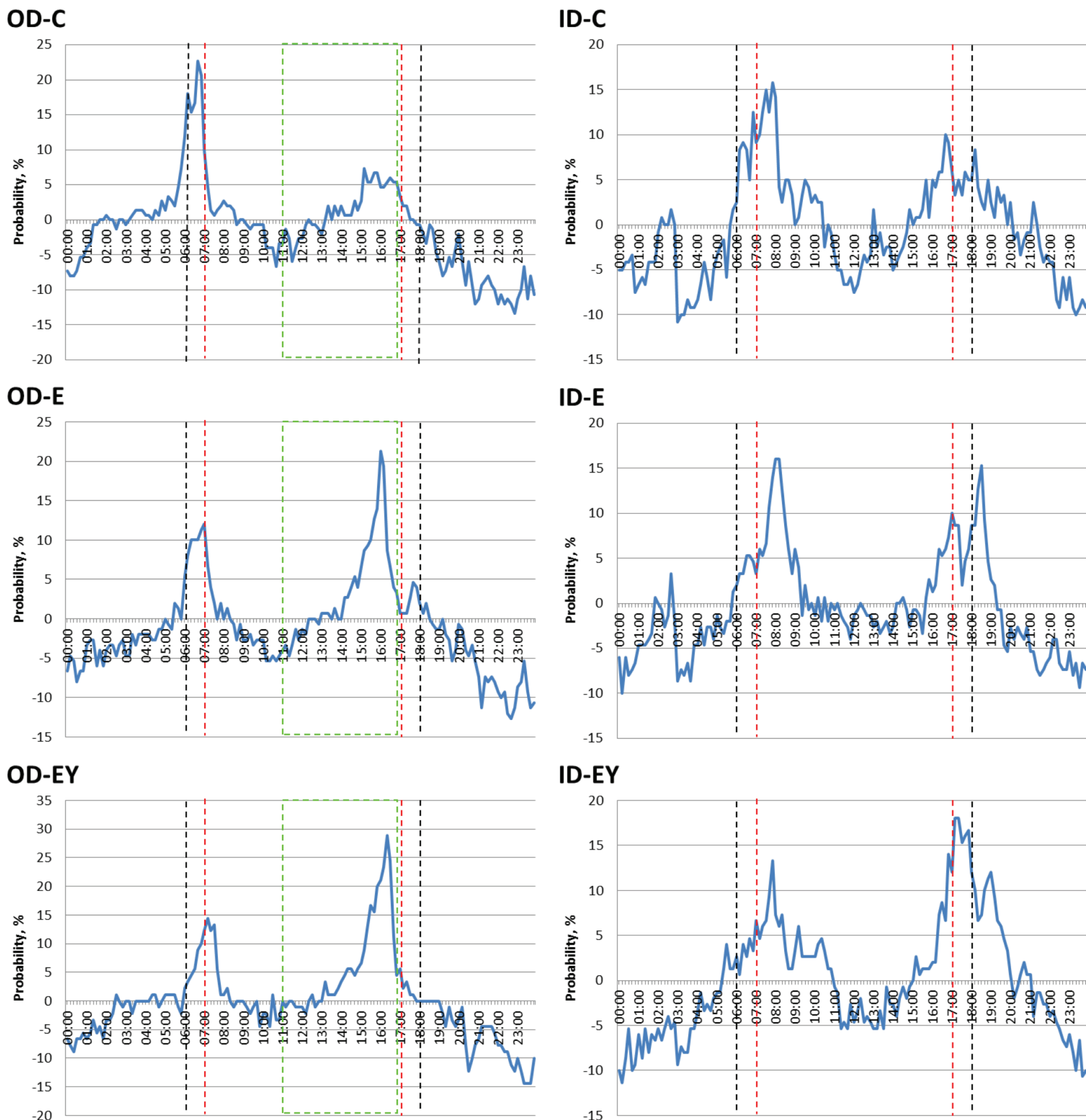

Fig. 1. Graphically visualized reticulorumen pH diurnal probability patterns
Trials and groups are represented in the upper left corner of the separate graph. Positive Y-axis values represent maximum pH values and negative values represent minimum values. The X-axis represents the time of the day in a 24 h (hh:mm) format with a smaller tick of 10 min. Black dotted lines represent the milking time, red dotted lines represent feeding with TMR, and the green dotted square is grazing time.

Discussion

To our knowledge, this is the first study that has used the Z-probability equation to predict the reticulorumen pH diurnal fluctuation. The results suggested that the probability for reticulorumen pH to be at maximum or minimum at a certain time had only minor differences across the treatment groups. The probability for maximum or minimum pH was more dependent on the feeding time rather than on feed supplements or the feeding strategy. In all treatment groups, the probability for maximum pH tended to be greatest within the morning and evening feeding, when the probability for minimum pH on average occurred at 5:33 ± 1:30 h after feeding. Our study results of ruminal pH diurnal fluctuation are consistent with those of Palmonari et al. (2010) and Salfer et al. (2018), where ruminal pH was highest just before feeding and declined for approximately 5 to 7 h thereafter, before gradually increasing again. Henk van Lingen et al. (2017) also reported that rumen fluid had pH 7.0 at feeding, decreased to pH 6.3 two hours after feeding, remained relatively constant for 5 h, and then increased to pH 6.7 at 10 h after feeding. Nordlund and Garrett (1994) suggested that the best time to take samples by rumenocentesis for SARA diagnostic is 5 to 8 h post-feeding in TMR-fed herds. According to the results from independent trials, we can conclude that cows' ruminal fermentation cycles are adapted to the feeding time.

Ruminal fermentation is performed by rumen microorganisms where the most important role belongs to rumen bacteria. Rumen bacteria consist of a vast amount of species where each species plays an important role in feed degradation and conversation into volatile fatty acids (VFA), an acceptable form of energy for a host animal. According to Kolver and De Veth (2002), the most abundant rumen bacteria, detected a few hours after feeding, were *Pseudobutyrivibrio spp.*, *Lactobacillus spp.*, *Selenomonas spp*, *Streptococcus spp.*, and *Prevotella spp.,* which rapidly convert carbohydrates into volatile fatty acids (VFA) and lactic acid. VFA and lactic acid strongly negatively correlate with rumen pH, which is responsible for increased probability for minimum reticulorumen pH. Kolver and Veth (2002) reported that in their study VFA concentration reached its maximum 3 h after feeding. The molar proportion of propionate reached a maximum of 22% in total VFA at 1.5 h after feeding. The proportion of butyrate steadily increased to a peak of 15% at 6 h after feeding and the molar proportion of acetate decreased from 68% to 62% over the first 1.5 h and then recovered after 3 h after feeding. Lactic acid was detected from 0.5 h to 4 h after feeding. The inflation of VFA and lactic acid in the first hours after feeding and their strong influence on rumen pH suggest that the Z-probability model of reticulorumen pH could be used to predict the diurnal fluctuation of VFA and lactic acid but the additional measurement is necessary.

Different feeding strategies could not necessarily result in different patterns of the diurnal reticulorumen pH fluctuation. The main difference between trials was that in trial OD along with TMR feeding a fresh pasture grass was included, while in trial OD the cows were only fed TMR. Fresh grass consists of soluble sugars and easily fermentable fiber, but according to the results, no significant differences in reticulorumen pH probabilities were observed between the treatments in general, or in the period when the cows were grazing. These observations could suggest that, while grazing, lactic acid is not produced in necessary quantities to induce a significant rumen pH decrease. However fresh grass is a good source of VFA production, whereas VFA are relatively weak acids and do not reduce the activity of fiber degrading bacteria (De Veth and Kolver, 2001). Besides, by comparing trial OD to trial ID, cow feeding behavior should be taken into account. The study of Pitt and Pell (1997) suggested that cow feed intake was not dependent on a feeding frequency. Cows fed 2 times and 12 times per day consumed the same amount of DM during the day. During our trials, the cows were always fed at the same time 2 times per day. The cows in trial OD instead of grazing fresh grass tended to wait for the main TMR meal, but further investigation of DM intake is necessary.

Forage supplementation with exoenzymes and active yeast did not give expected significant results to reticulorumen pH. It is possible that neither exoenzymes nor active east may have abilities to affect reticulorumen pH, but more likely that the z-probability equation and its adaptation to the diurnal reticulorumen pH fluctuation are not suitable to find meaningful differences between treatments. Only the small positive effect of yeast was observed in group ID–EY when the time length of probability for maximum reticulorumen pH was longest. A meta-analysis prepared by Desnoyers et al. (2009) revealed that yeast supplementation increased rumen pH (+0.03 on average) and rumen volatile fatty acid concentration (+2.17 mM on average), tended to decrease rumen lactic acid concentration (–0.9 mM on average) and increased total-tract organic matter digestibility (+0.8% on average).

By comparing the results with other studies where the circadian rhythm of reticulorumen pH was analyzed, the results of both trials in group C were similar to those of Denwood et al. (2018) and Josson et al. (2019) where the reticulorumen pH was highest in the first half of the day. Our results contribute to the research where reticulorumen pH values started increasing after 22:00 h (Antanaitis et al., 2016), explaining a pH increase due to saliva excretion during rumination at the nighttime (Josson et al., 2019).

Conclusions

According to our results, we can conclude that the Z-probability equation is acceptable for the prediction

of reticulorumen pH diurnal fluctuation. The diurnal fluctuation of reticulorumen pH had high chances to be at maximum two times per day: in the morning and in the evening; minimum pH values have the greatest probability to occur at midnight. TMR supplementation with exoenzymes and active yeast could affect the daily time of the maximum pH values to occur, but has no effect on time for the minimum pH values. This research also has practical benefits. Using reticulorumen pH probability patterns, cow feeding strategies and rumen sampling strategies could be improved.

References

1. Adesogan A., Arriola K., Jiang Y., Oyebade A., Paula E., Pech-Cervantes A., Vyas D. Symposium review: Technologies for improving fiber utilization. Journal of Dairy Science. 2019. T. 102. P. 5726 – 5755.
2. Alzahal O., Kebreab E., France J., Mcbride B. A Mathematical Approach to Predicting Biological Values from Ruminal pH Measurements. Journal of Dairy Science. 2007. T. 90. P. 3777 – 3785.
3. Antanaitis R., Žilaitis V., Juozaitienė V., Stoškus R., Televičius, M. Changes in reticulorumen content temperature and pH according to time of day and yearly seasons. Polish Journal of Veterinary Sciences. 2016. T. 19. P. 771 – 776.
4. Deng W., Xi D., Mao H., Wanapat M. The use of molecular techniques based on ribosomal RNA and DNA for rumen microbial ecosystem studies: A review. Molecular Biology Reports. 2008. T. 35. P. 265 – 274
5. Desnoyers M., Giger-Reverdin S., Bertin G., Duvaux-Ponter C., Sauvant D. Meta-analysis of the influence of Saccharomyces cerevisiae supplementation on ruminal parameters and milk production of ruminants. Journal of Dairy Science. 2009. T. 92. P 1620-1632.
6. De Veth M. Kolver E. Diurnal Variation in pH Reduces Digestion and Synthesis of Microbial Protein when Pasture is Fermented in Continuous Culture. Journal of Dairy Science. 2001. T. 84. P. 2066 – 2072.
7. Denwood M., Kleen J., Jensen D., Jonsson N. Describing temporal variation in reticuloruminal pH using continuous monitoring data. Journal of Dairy Science. 2018.T 101. P. 233-245.
8. Fonty G., Chaucheyras-Durand F. Effects and modes of action of live yeasts in the rumen. Biologia. 2006. T. 61. P. 741 – 750.
9. Jonsson N., Kleen J., Wallace R., Andonovic I., Michie C., Farish M., Denwood M. Evaluation of reticuloruminal pH measurements from individual cattle: Sampling strategies for the assessment of herd status. The Veterinary Journal. 2019. T. 243. P. 26 – 32
10. Kleen J., Hooijer G., Rehage J., Noordhuizen J. Subacute Ruminal Acidosis (SARA): A Review. Journal of Veterinary Medicine Series A. 2003. T. 50. P. 406 – 414.
11. Kolver E., De Veth M. Prediction of Ruminal pH from Pasture-Based Diets. Journal of Dairy Science. 2002. T. 85. P. 1255 – 1266.
12. Nordlun K. V., Garrett E. F. Rumenocentesis: a technique for collecting rumen fluid for the diagnosis of subacute rumen acidosis in dairy herds. The Bovine Practitioner. 1995. T. 28. P. 109 – 112.
13. Palmonari A., Stevenson D., Mertens D., Cruywagen C., Weimer P. PH dynamics and bacterial community composition in the rumen of lactating dairy cows. Journal of Dairy Science. 2010. T. 93. P. 279 – 287.
14. Pitt R., Pell A. Modeling Ruminal pH Fluctuations: Interactions Between Meal Frequency and Digestion Rate. Journal of Dairy Science. 1997. T. 80. P. 2429 – 2441.
15. Plaizier J. C., Krause D. O., Gozho G. N, McBride B. W. 2008. Subacute ruminal acidosis in dairy cows: The physiological causes, incidence and consequences. The Veterinary Journal. 2008. T. 176. P. 21 – 31.
16. Salfer I., Morelli M., Ying Y., Allen M., Harvatine K. The effects of source and concentration of dietary fiber, starch, and fatty acids on the daily patterns of feed intake, rumination, and rumen pH in dairy cows. Journal of Dairy Science. 2018. T. 101. P. 10911 – 10921.
17. Van Lingen H., Edwards J., Vaidya J., Van Gastelen S., Saccenti E., Van Den Bogert B., Dijkstra J. Diurnal Dynamics of Gaseous and Dissolved Metabolites and Microbiota Composition in the Bovine Rumen. Frontiers in Microbiology. 2017. T. 8. P. 425.
18. Whitlock M. Combining probability from independent tests: The weighted Z-method is superior to Fisher's approach. Journal of Evolutionary Biology. 2005. T. 18. P. 1368 – 1373.
19. Zebeli Q., Aschenbach J., Tafaj M., Boguhn J., Ametaj B., Drochner W. Invited review: Role of physically effective fiber and estimation of dietary fiber adequacy in high-producing dairy cattle. Journal of Dairy Science. 2012. T. 95. P. 1041 – 1056.
20. Zebeli Q., Tafaj M., Weber I., Dijkstra J., Steingass H., Drochner W. Effects of varying dietary forage particle size in two concentrate levels on chewing activity, ruminal mat characteristics, and passage in dairy cow. Journal of Dairy Science. 2007. T. 90. P. 1929 – 1942.

Received 28 January 2020
Accepted 2 July 2020

Influence of Heat Stress and Some Related Physiological Indicators on the Content of Long-Chain Fatty Acids in the Milk of Holstein-Friesian Cows

Toncho Penev[1], Nikolina Naydenova[2], Dimo Dimov[1], Ivaylo Marinov[1]
[1]*Department of Applied Ecology and Animal Hygiene, Faculty of Agriculture, Trakia University, Bulgaria*
[2]*Department of Animal Husbandry – Ruminants and Dairy Farming, Faculty of Agriculture, Trakia University, Bulgaria*

Keywords: *heat stress, temperature-humidity index, rectal temperature, long-chain fatty acids, Holstein-Friesian cows.*

Abstract. *The aim of the research was to study the effect of heat stress (HS) and associated changes in the rectal temperature and the respiratory rate on long-chain fatty acid (LCHFA) content in the milk of Holstein-Friesian cows. The study included 22 cows on different parities studied in two periods: under thermo-neutral environment conditions (May 2018) and under heat stress (August 2018). The fatty acid content of milk was determined using a chromatograph by the method of Rose-Gottlieb. It was found that HS in dairy cows leads to changes in the content of some LCHFAs (C17:0; C18:0; C18:2 and C18:3) in milk fat. Under conditions of moderate HS (temperature-humidity index over 79), a certain decrease in the content of C17:0 was reported, while in the other three LCHFAs, an increase in their content in milk fat was reported to varying degrees. The strongest effect of HS was reported on the content of C18:0, which was proportional to the levels of HS. With an increasing rectal temperature, an increase in the content of C18:0 was reported, the increase being most substantial at a rectal temperature above 39.5°C.*

Introduction

In the last few decades of the 19th century, we have witnessed dramatic changes in the Earth's climate associated with warming (Renna et al., 2010). Such trends are reported in different climatic zones, including the temperate continental. Milk cows are very sensitive to climate change and especially to elevated temperatures. Heat stress in dairy cows is usually associated with activation of certain thermoregulatory mechanisms to overcome the hyperthermia and maintain vital functions of the animal and, as a consequence, reduction of their productivity (Nardone et al., 2006). Exposure to heat stress can dramatically change both the quantity and composition of ruminant milk (Renna et al., 2010). Protein in milk has been found to decrease significantly under conditions of heat stress (Giustini et al., 2007; Kamiya et al., 2005). Studies by a number of authors have shown different trends in the milk fat content of cows' milk under heat stress conditions (Giustini et al., 2007; Bouraoui et al., 2002; Sevi et al., 2001; Lacetera et al., 1996). Some authors do not find changes in the amount of fat in cow's milk at HS (Roman-Ponce, 1977; Knapp and Grummer, 1991; Lacetera et al., 2003), while others report a significant decrease (Bouraoui et al., 2002; Hammami et al., 2015; Hill and Wall 2015). However, surveys that have studied the effect of HS on the fatty acid profile of milk are few, and the results between the authors are controversial (Lacetera et al., 2003). The fatty acid profile of milk has been found to be a potential indicator of energy balance in cows (Bastin et al., 2012). HS leads to certain physiological changes in dairy cows such as increased respiratory rate, increased heart rate and increased body temperature (Kadzere et al., 2002; Wheelock et al., 2010), but the most prominent change is the reduced intake of dry matter (West, 2003), which, together with the efforts for cooling, results in consumption of much energy and metabolic changes in the body of dairy cows (Bernabucci et al., 1999). According to Chilliard et al. (2000) and Parodi (2004), the content of LCHFAs after C16:0 in milk is a consequence of metabolism in the body and they are not synthesized de novo in the mammary gland of cows. According to the authors, all processes related to metabolism in the body under metabolic changes can cause a number of alterations in the percentage of LCHFAs. Most of the LCHFAs in milk have different effects on human health. Palmitic acid (C 16:0) together with some medium-chain saturated fatty acids (C12:0 and C14:0) are considered to be major factors in the development of cardiovascular diseases in humans (Kaylegian and Lindsay, 1995; Jensen, 2002). On the other hand, some unsaturated LCHFAs, such as oleic (C18:1), linoleic (C18:2) and linolenic (C18:3), have a positive effect on human health (Simopoulos, 2002). HS, leading to changes in the content of these fatty acids in milk, is of interest both in relation to the metabolic changes in the body of cows and respectively their welfare, and in relation to the qualities of produced milk as healthy food for humans.

The aim of the study was to investigate the effect of HS and associated changes in the rectal temperature and the respiratory rate on the percentage of

Correspondence to Toncho Penev, Department of Applied Ecology and Animal Hygiene, Faculty of Agriculture, Trakia University, 6000 Stara Zagora, Bulgaria
E-mail: tonchopenev@abv.bg

LCHFAs – isomargaric (C17:ISO), margaric (C17:0), stearic (C18:0), oleic (C18:1), linoleic (C18:2) and linolenic (C18:3) – in milk fat in Holstein-Friesian cows.

Material and Methods

The study was conducted at a cattle farm with Holstein-Frisian cows near Karnobat, southeastern Bulgaria, in 2018. Cows were housed in a semi-open free stall dairy barn, fed year-round ad libitum with a total mixed ration at the following composition for a cow per day in kg: forage – 14 kg, concentrates – 14 kg, and vitamin and mineral supplements – 0.39 kg. The content of the forage expressed as a percentage of the total amount is as follows: corn silage – 77.15%, alfalfa hay – 12.14%, and straw – 10.71%. The content of concentrates expressed as a percentage of their total quantity is as follows: maize grain (ground) – 35.74%, wheat grain (ground) – 21.42%, barley grain (ground) – 7.14%, soybean meal – 11.42%, sunflower meal – 19.28%, and palm oil – 5%. The study included all cows calved between first April and tenth May 2018, thus excluding the effect of the lactation period on the fatty acid content of milk. The included cows were at first (9 cows), second (6 cows) and third to fifth (7 cows) parity or a total of 22 cows. To test for fatty acid content, individual milk samples with a volume of 100 mL from the morning and the evening milking were taken from each cow included in the study once in May and once in August. The daily milk yield as well as the content fat and protein percentage of the cows were taken from their official monthly milk performance records (for May and August).

Extraction of milk fat was performed by the Rose-Gottlieb method using diethyl ether and petroleum ether (Methodenbuch, Bd. VI VDLUFA-Verlag, Darmstadt, 1985). The solvents were then evaporated on a vacuum rotary evaporator. Sodium methylate (CH3ONa) was used to prepare the fatty acid methyl esters. The fatty acid content of milk fat was determined by a “Clarus 500” gas chromatograph with a flame-ionization detector and a “ThermoScientist” column (60 m, ID 0.25 mm, film: 0.25 μm).

To measure the heat stress, a temperature-humidity index (THI) was estimated using a “Kestrel” automatic measuring instrument. Reporting of THI was carried out in the cows’ housing premises once a day, at 3 pm, with examination of the physiological parameters of each cow: the rectal temperature and the respiratory rate per minute. The rectal temperature was measured by a digital thermometer in degrees of Celsius. The respiratory rate was reported by visual observation and recording of the movement of the chest for a period of one minute according to the method of Zimbelman et al. (2009).

For better approximation factors object of the study were presented in classes as follows:

THI is presented in three classes according to the proposed THI scale by Armstrong (1994), respectively: Class 1 – THI up to 72; Class 2 – 72 to 79 (mid-moderate heat stress conditions); and Class 3 – THI above 79 (moderate heat stress conditions).

The rectal temperature of the cows, respectively: Class 1 – up to 38.5°C; Class 2 – from 38.5 to 39°C; Class 3 – from 39 to 39.5°C; and Class 4 – above 39.5°C.

Respiratory rate per minute: Class 1 – up to 40 per minute; Class 2 – 40 to 45 per minute; Class 3 – 45 to 55 per minute; and Class 4 – over 55 movements per minute.

For basic statistical processing of the data, a package MS Excel was used, and for obtaining the average values, errors, and analysis of variance, the corresponding modules of STATISTICA of StatSoft was employed (Copyright 1990–1995 Microsoft Corp.)

The following model was used to evaluate the influence of controlled factors on the content of long-chain fatty acids in milk:

$$Y_{ijkl} = \mu + THI_i + RT_j + B_k + e_{ijkl}$$

Where: Y_{ijkl} is the dependent variable (each of the fatty acids studied), μ is the mean effect, THI_i is the effect of THI (classes), RT_j is the effect of rectal temperature (classes), B_k is the effect of respiratory movements per minute (classes) and e_{ijkl} is the random residual effect.

By analysis of variance (ANOVA) for the model were obtained by classes of fixed factors the means of least squares (LSM).

Results

Table 1 presents the reported THI averages for the two calendar months, with a significant difference of 73.4 and 79.1, respectively. The variation in THI values for May corresponds to conditions from optimal to mid-moderate heat stress, THI 71.2 and THI 76.2, respectively. For August, the reported minimum-maximum values of THI were significantly higher corresponding to the conditions from mid-moderate heat stress (THI 77.1), to conditions of moderate heat stress (THI 81.5).

Table 1 also presents the average Test day records for milk yield, fat and protein percentage, and the average values of the reported physiological parameters of the examined cows for the two months of the study (May and August). The cows included in the study had a high average daily milk yield of 39.92 kg, a high fat content (4.13%) and a low protein content of 2.88%. A slight difference in the average TD performance values for the two months was observed. The average milk yield and fat percentage had lower averages for August compared with those for May but without a significant difference. There was a slight increase in the TD protein percentage, and although the difference was significant, it was very small – by only 0.1%.

Table 1. Average values and variation of productive traits for Test day, physiological indicators and temperature-humidity index by months of reporting

Indicator	May, n = 22			August, n = 19		
	x ± SE	min	max	x ± SE	min	max
Milk yield, kg	41.12 ± 2.52 n.s.	19.5	60.1	38.6 ± 2.19 n.s.	23.7	52.9
Fat %	4.31 ± 0.22 n.s.	2.70	5.87	3.94 ± 0.21 n.s.	2.28	5.56
Protein %	2.84 ± 0.02**	2.60	3.00	2.94 ± 0.02**	2.77	3.15
Rectal temperature, ºC	38.55 ± 0.09***	37.3	39.2	39.27 ± 0.10***	38.2	39.9
Respiratory rate, number/min	39.27 ± 1.57***	24.0	56.0	54.95 ± 1.91***	36.0	682.0
Temperature-humidity index	73.4 ± 0.29***	71.2	76.2	79.1 ± 0.49***	77.1	81.5

n.s.– has no significant effect.
*Significance at P < 0.05. **Significance at P < 0.01. ***Significance at P < 0.001.

The average rectal temperature of cows increased from 38.55ºC in May to 39.27ºC in August, and the respiratory rate per minute increased from 39.27 to 54.95, respectively. Table 2 presents the average values and the variation in the content of long-chain fatty acids in milk fat. Under the moderate temperature conditions for May, the values of C18:0 and C18:3 were lower, 8.46% and 0.42%, respectively, and under conditions of low and moderate HS in August their values were increased to 9.38% and 0.54%. With LCHFA C17:0, the trend was opposite, i.e., the values decreased slightly under conditions of HS, from 3.46% to 3.21%.

Table 3 presents an analysis of variance for the influence of THI, rectal temperature and respiration rate on the contents of LCHFAs in the milk. The THI affected significantly four of the six studied fatty acids: C17:0; C18:0; C18:2 and C18:3. The rectal temperature had a significant effect only on the C18:0 content.

Table 2. Mean values (in %) and variation in the content of long-chain fatty acids in milk fat by months of reporting

LCHFAs	May, n = 22			August, n = 19		
	x ± SE	min	max	x ± SE	min	max
C17:ISO	0.55 ± 0.02 n.s.	0.26	0.72	0.58 ± 0.02 n.s.	0.37	0.76
C17:0	3.46 ± 0.09*	2.81	4.58	3.21 ± 0.06*	2.58	3.57
C18:0	8.49 ± 0.14***	7.10	9.65	9.38 ± 0.14***	8.16	10.33
C18:1	25.19 ± 0.56 n.s.	20.88	29.59	26.23 ± 0.34 n.s.	23.46	28.77
C18:2	2.60 ± 0.07 n.s.	2.04	3.18	2.60 ± 0.06 n.s.	2.01	2.95
C18:3	0.42 ± 0.03**	0.14	0.68	0.54 ± 0.02**	0.35	0.67
Total	40.33			42.54		

n.s.– has no significant effect.
*Significance at P < 0.05. **Significance at P < 0.01. ***Significance at P < 0.001.

Table 3. Analysis of variance for the influence of temperature-humidity index, rectal temperature and respiration rate on the contents of long chain fatty acids

Sources of variation	Degrees of freedom (n–1)	C17:ISO	C17:0	C18:0	C18:1	C 18:2	C18:3
		F P	F P	F P	F P	F P	F P
Total for the model	8	1.84 n.s.	1.64 n.s.	2.68 *	1.53 n.s.	1.85 n.s.	2.39 *
Temperature-humidity index	2	2.69 n.s.	4.27 *	6.61 **	2.35 n.s.	3.57 *	3.46 *
Rectal temperature	3	0.80 n.s.	1.31 n.s	3.30 *	1.23 n.s.	0.85 n.s.	2.00 n.s.
Respiratory rate	3	1.83 n.s.	1.18 n.s.	1.00 n.s.	1.73 n.s.	2.18 n.s.	1.01 n.s.
Error	32						

n.s.– has no significant effect.
*Significance at P < 0.05. **Significance at P < 0.01. ***Significance at P < 0.001.

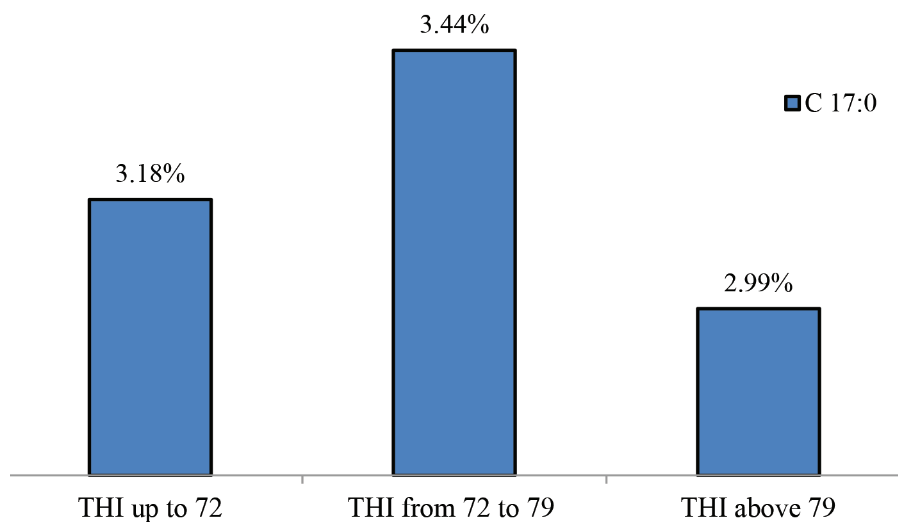

Fig.1. LS means for C17:0 content depending on the THI

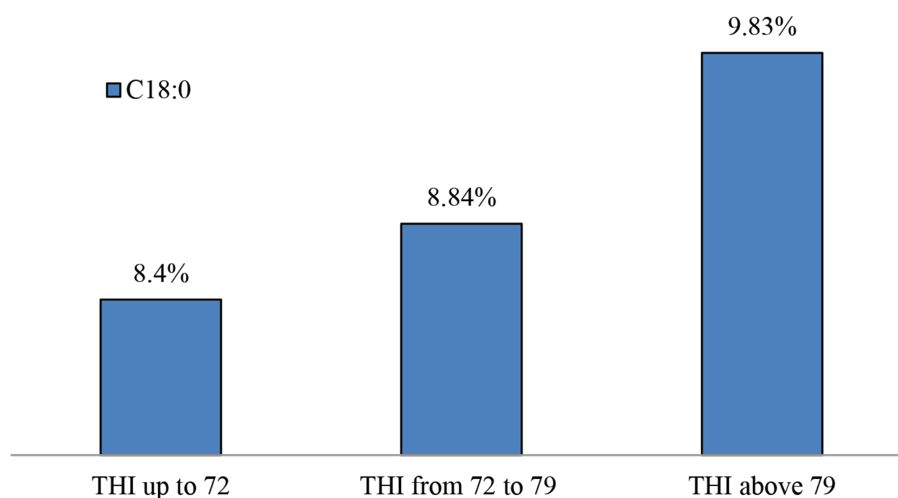

Fig. 2. LS means for C18:0 content depending on the THI

Fig. 1 shows the LS mean values for C17:0 content of milk fat, depending on the THI values. The highest percentage (3.44%) of C17:0 in milk fat was recorded at the THI values from 72 to 79 corresponding to mild heat stress. With an increase in the THI values above 79 (moderate heat stress), the percentage C17:0 content decreased to 2.99%.

Fig. 2 shows the LS mean values for the C18:0 content at different THI levels. At the THI up to 72, the content of C18:0 was 8.4%, at the THI from 72 to 79, it was 8.84, and at the THI above 79, it increased to 9.83%.

Fig. 3 presents the LS mean values of C18:0 depending on the rectal temperature. At values of the rectal temperature up to 38.5°C, the values of C18:0 were 8.58% and increased to 10% at a rectal temperature above 39.5°C.

Fig. 4 shows the LS means for the C18:2 content depending on the THI levels. With the increase in the THI values from mild (up to 72) to moderate HS (THI from 72 to 79), the increase in the content of this fatty acid was almost 0.4%.

Fig. 5 presents the LS mean values for the content of C18:3, depending on the THI. At the THI up to 72, the amount of linolenic acid (C18:3) was 0.36%, at the THI from 72 to 79, it was 0.47%, and at the THI over 79, it was 0.56%. The tendency of increasing content of C18:3 with the increase of the THI value was clearly evident here.

Discussion

Similarly to our data presented in Table 1, Dimov et al. (2017) also reported THI risk values for the region of southern Bulgaria. The authors indicate that, during the summer season, values determining conditions for mild to moderate heat stress in dairy cows were reported, namely, average daily values of the THI over 75. There was also a certain risk of such

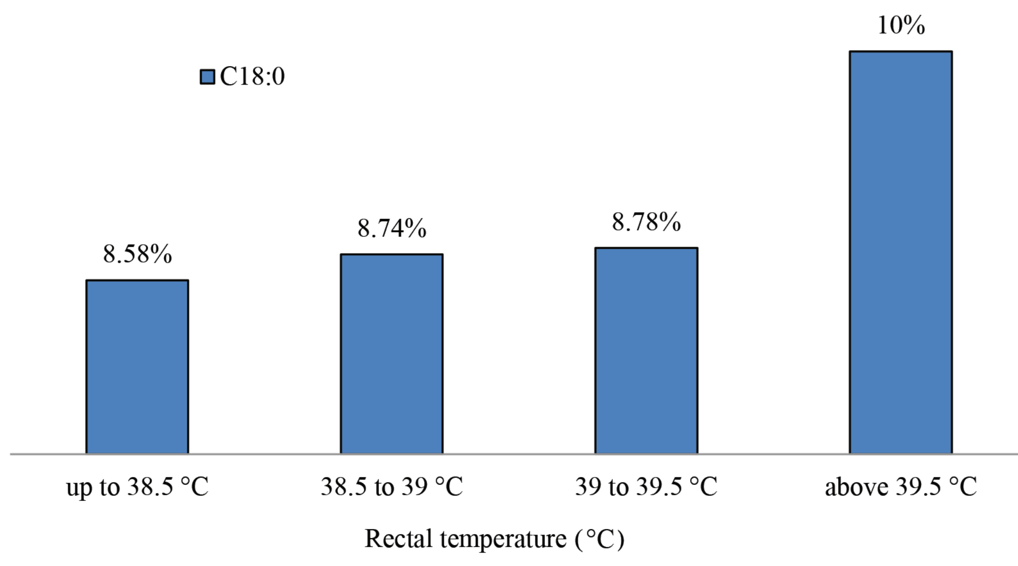

Fig. 3. LS means for C18:0 content depending on the rectal temperature

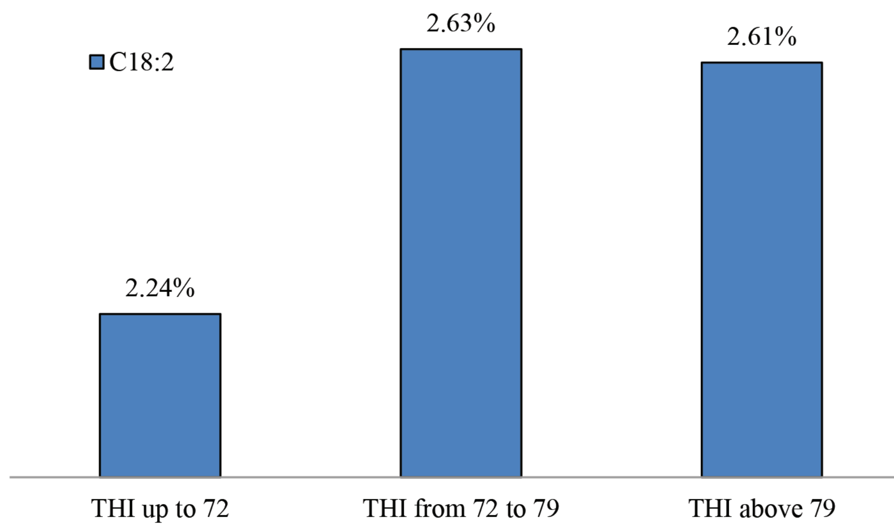

Fig. 4. LS means for C18:2 content depending on the THI

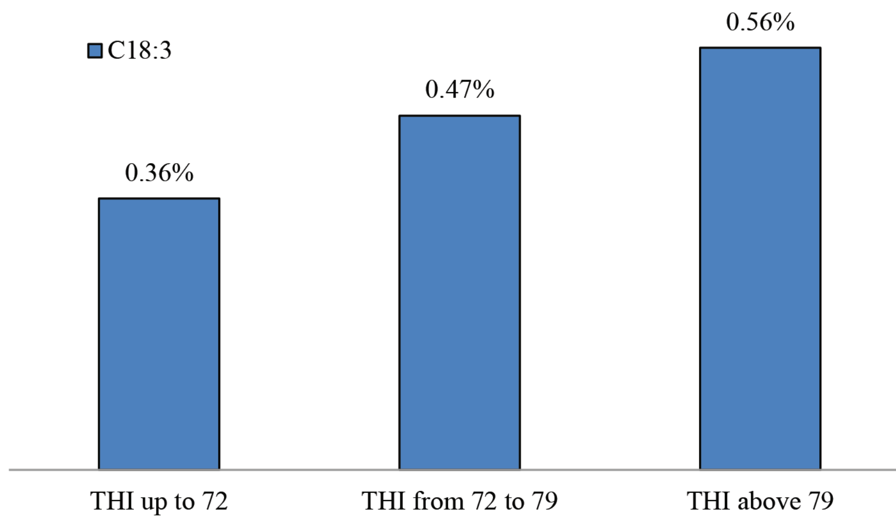

Fig. 5. LS means for C18:3 content depending on the THI

conditions in spring and autumn. It can be said that, with respect to the main productive traits of the cows included in the study, no significant differences were observed for the two calendar months and the reported THI values, although the trend showed a slight decrease in TD milk yield and TD fat percentage in milk and a slight increase in the TD protein percentage under mild to moderate HS in August (Table 1). There were no unidirectional data on the effect of HS on milk composition reported by other authors as well. Bouraoui et al. (2002) also found a decrease in milk yield and a fat percentage under the influence of HS, while Smith et al. (2013) found an increase in fat content in milk under mild HS (THI over 68). These differences may also be due to different levels of HS in the studies by different authors.

In respect of the two physiological indicators, i.e., rectal temperature and respiratory rate, the results in Table 1 show that in May at the optimal to slightly risky THI values significantly lower values for the rectal temperature and the respiratory rate per minute were reported. Similar changes in the rectal temperature values and the respiratory rate in cows under the influence of HS were also found by Bouraoui et al. (2002). According to Atkins et al. (2018), the respiratory rate increases by 1.95 breaths per minute at the THI below 70 and by 3.18 breaths per minute at the THI over 70. This explains the differences in the respiratory rate per minute in our study.

Significant differences in the content for the two months of studying were reported in three studied LCHFAs: C17:0, C18:0 and C18:3. Our results for C17:0 and C18:0 were consistent with the studies of Hammami et al. (2015). Under the conditions of HS, Liu et al. (2016) also found an increase in C18:3 content in milk fat. The other LCHFAs showed very small (C17:ISO and C18:1) or no difference (C18:2) under both optimal and HS conditions. Our results regarding the C18:1 greatly differed from the results of Liu et al. (2016). The authors found a significant increase in the content of this fatty acid under HS conditions lasting 4 days. According to Liu et al. (2016), under HS conditions, LCHFAs are increased of the occurred lipolysis of the adipose tissue.

The data presented in Table 2 show that the total amount of LCHFAs reported in August (conditions of mild to moderate HS) was higher by 2.21%, compared with their total in May, under optimal temperature conditions. Renna et al. (2010) also found an increase in the amount of LCHFAs under HS conditions.

The data in Table 3 show that the THI was the factor that significantly influenced four of the six studied fatty acids: C17:0; C18:0; C18:2 and C18:3. In regard of the two physiological indicators, the respiratory rate had no significant effect on the studied LCHFAs, and the rectal temperature had a significant effect only on the C18:0 content.

Similarly to our results in Figure 1, Hammami et al. (2015) also found a decrease in the content of margaric acid (C17:0) at the THI values above 62.

The THI had the highest significance effect ($P < 0.01$) on the C18:0 content (Figure 2). These data partly confirm the findings of Hammami et al. (2015), according to which C18:0 and C18:1 were the fatty acids affected by HS. Contrary to the results reported reported by Hammami et al. (2015), C18:1 was not significantly affected by HS levels reported in our conditions. This was probably due to the difference in the THI values. In our study, the reported THI values were around and above 79, while in the study of Hammami et al. (2015), the highest THI value was 75. A more detailed analysis of the data presented in Figure 2 shows that with the increase in THI values, the percentage of C18:0 in milk fat also increased. These results confirm those of Barber et al. (1997). According to the authors, when cows were subjected to a negative energy balance, as observed under HS, a higher ratio of C18:0 and C18:1 cis-9 was found. In other words, the presence of moderate heat stress led to lipolysis, which caused an increase in the C18:0 content. Based on our results, we consider that the most sensitive change in cows under HS conditions was reported in stearic acid (C18:0) compared with the other analyzed LCHFAs.

From the LS figures shown in Figure 2, it can be seen that the increase in C18:0 content in cow's milk at the THI up to 72 and from 72 to 79 is only 0.44%. When the THI increased above 79 (conditions of moderate HS), the content of this fatty acid increased by almost 1%. The increase in stearic acid values reflected the metabolic changes occurring in the body of the cows, which affected the fatty acid content of the milk they produce. Bandaranayaka and Holmes (1976) found that a high ambient temperature of 30°C was associated with a statistically significant increase ($P < 0.05$) of C18:0 content in milk fat.

In support of the above thesis that the amount of C18:0 in milk of dairy cows is an objective indicator of moderate heat stress presence, a significant effect ($P < 0.05$) of the rectal temperature on the content of this fatty acid was also reported (Table 3).

A clear trend for an increase in content of C18:0 with the increasing rectal temperature was observed. As the THI values increased, an increase in the cows' rectal temperature was also observed (Srikandakumar and Johnson, 2004). According to (Ammer et al., 2016), the normal body temperature of cows varied between 38°C and 39.2°C. Our study found that the increase in C18:0 content in milk fat in cows within the physiological norm for the rectal temperature (up to 39.5°C) was minimal, i.e., 0.2%. However, an increase in the rectal temperature of cows above 39.5°C was associated with a significant increase in the percentage of C18:0 in milk fat, possibly due to increased lipolysis in the body due to reduced dry matter intake (Barber et al., 1997; West, 2003; Wheelock et al., 2010; Bouraoui et al., 2002; Rhoads et al., 2009).

These results confirm the studies of Liu et al. (2016), who found an increase in the content of this fatty acid by 4.1 mg/g of milk fat under HS within

the THI range between 72 and 84. In our study, it was found that increasing the THI values above 79 did not maintain the tendency of increasing the C18:2 content, as reported for C18:0. There was even a slight decrease in its content by 0.02% in moderate heat stress (THI over 79), compared with cows under mild HS (THI 72 to 79). In a study by Nantapo et al. (2014) with advancing of lactation, the content of linoleic acid (C18:2) in the milk decreased. The increase we found presented in Figure 4 shows that the influence of HS was very strong, overcoming the physiologically expected trend for lowering. In the studies of Liu et al., (2016), the C18:2 content increased in absolute values, but the authors did not consider how this fatty acid content changed at different levels of HS. Based on the results of our study it follows that lipolysis occurring in the body of cows under moderate HS most considerably affected the content of stearic acid. According to Hammami et al. (2015), the most substantial change in the fatty acid composition of milk under conditions of HS concerns mainly C18:1 cis-9. The differences between our results and those of other authors may also be due to the differences in the feeding strategy of the cows and their daily milk yield. In the study of Hammami et al. (2015), the average daily milk yield of cows reached 23.43 kg per day, while in our study it was significantly higher, i.e., - 39.92 kg per day. Cows with higher milk yield reacted with occurrence of symptoms of HS (increased respiratory rate and higher rectal temperature) at lower values of the THI due to their higher sensitivity to HS (Bernabucci et al., 2014). As cow productivity increases, the metabolic heat they produce increases (Purwanto et al., 1990), which increases their sensitivity to HS (Kadzere et al., 2002). The increase was 0.39% at the THI from 72 to 79, and 0.37% at the THI above 79, relative to thermo-neutral conditions (THI below 72).

According to Liu et al. (2016), HS induced an increase in the content of LCHFAs in milk. Our study on the content of C18:3 in milk confirmed the results obtained in studies of Liu et al., (2016), finding that the content of this fatty acid increased proportionally to the increase of heat stress. According to Nantapo et al. (2014), similarly to linoleic (C18:2), the content of linolenic acid (C18:3) should normally also decrease as lactation progresses. In our study in August, cows were in more advanced stages of lactation (third or fourth lactation month) compared with May (first or second) and a decrease should be reported in the content of these fatty acids. Higher values reported in August indicate that HS had a significant effect on the content of long-chain unsaturated fatty acids, which outweighs the physiological effect of the lactation stage.

Linolenic acid (C18:3) has a proven beneficial effect in the prevention of heart disease and improved immune response in humans (Muchenje et al., 2009; Palladino et al., 2010). The study found that heat stress caused an increase in the amount of long-chain fatty acids in cow's milk, especially stearic, oleic and linolenic acid. Increasing or maintaining the levels of unsaturated fatty acids in milk specific for early lactation, under the influence of HS, would be healthier for people who consume it. However, heat stress has a detrimental effect on the welfare and health of cows in many aspects, which, despite the above-mentioned positive effect on human health, requires that measures be taken to eliminate or mitigate it.

Conclusion

The heat stress in dairy cows results in changes in the content of some long-chain fatty acids (C17:0; C18:0; C18:2 and C18:3) in milk fat. Under conditions of moderate heat stress (THI over 79), a slight decrease in the content of C17:0 was reported, while in the other three LCHFAs, an increase of their content in the milk fat in varying degrees was found. The strongest influence of heat stress was reported on the C18:0 content, which was proportional to the levels of HS. As the rectal temperature increases, an increase in the C18:0 content is observed, with the increase being most substantial at a rectal temperature above 39.5°C.

References

1. Ammer S., Lambertz C., Gauly M. (2016). Comparison of different measuring methods for body temperature in lactating cows under different climatic conditions. J. Dairy. Res., 83: 165–172.
2. Armstrong D. V., 1994. SYMPOSIUM: Nutrition and heat stress. Heat Stress Interaction with Shade and Cooling. J Dairy Sci 77:2044-2050.
3. Atkins, I. K., N. B. Cook, M. R. Mondaca, C. Y. Choi, 2018. Continuous respiration rate measurement of heat-stressed dairy cows and relation to environment, body temperature, and lying time. Transactions of the ASABE Vol. 61(5): 1475-1485. https://doi.org/10.13031/trans.12451
4. Bandaranayaka D. D. and C. W. Holmes, 1976. Changes in the composition of milk and rumen contents in cows exposed to a high ambient temperature with controlled feeding. Trop. Anim. Filth Prod. (1976) 8, 38-46.
5. Barber MC, Clegg RA, Travers MT, Vernon RG, 1997. Lipid metabolism in the lactating mammary gland. Biochim Biophys Acta. 1997 Aug 16;1347(2-3):101-26
6. Bastin, C., D. P. Berry, H. Soyeurt, and N. Gengler. 2012. Genetic correlations of days open with production traits and contents in milk of major fatty acids predicted by mid-infrared spectrometry. J. Dairy Sci. 95:6113–6121.
7. Bernabucci U., Biffani S., Buggiotti L., Vitali A., Lacetera N., Nardone A. (2014). The effects of heat stress in Italian Holstein dairy cattle. J. Dairy. Sci., 97: 471–486.
8. Bernabucci, U., Bani, P., Ronchi, B., Lacetera, N. & Nardone, A. Influence of short- and long-term exposure to a hot environment on rumen passage rate and diet digestibility by Friesian heifers. J Dairy Sci 82, 967–73 (1999).
9. Bouraoui, R., Lahmar, M., Majdoub, A., Djemali, Mn. & Belyea, R. The relationship of temperature-humidity index with milk production of dairy cows in a Mediterranean climate. Anim. Res. 51, 479–491 (2002).
10. Chilliard, Y., Ferlay, A., Mansbridge, R. M. & Doreau, M. Ruminant milk fat plasticity: nutritional control of saturated, polyunsaturated, trans and conjugated fatty acids. Ann. Zootech. 49, 181–205 (2000).

11. German J.B. and Dillard C. J. (2006). Composition, structure and absorption of milk lipids: a source of energy, fat-soluble nutrients and bioactive molecules. Crit Rev Food Sci Nutr.,46:57–92.
12. Giustini, L., Acciaioli, A., Pianaccioli, L., Surace, R. and O. Franci (2007) Thermic stress effect on the main qualitative parameters of milk produced in the Mugello. Scienza e Tecnica Lattiero-Casearia 58(6): 383-402.
13. Dimov D., I. Marinov, T. Penev, Ch. Miteva, Zh. Gergovska. 2017. Influence of temperature-humidity index on comfort indices in dairy cows. SYLWAN., 161(6), 68 - 85.
14. Hammami H, Vandenplas J, Vanrobays ML, Rekik B, Bastin C, Gengler N, 2015. Genetic analysis of heat stress effects on yield traits, udder health, and fatty acids of Walloon Holstein cows. J Dairy Sci. 2015 Jul; 98(7):4956-4968.
15. Hill, D. L. & Wall, E. Dairy cattle in a temperate climate: the effects of weather on milk yield and composition depend on management. Animal 9, 138–49 (2015).
16. Jensen R. G., 2002. Invited Review: The Composition of Bovine Milk Lipids: January 1995 to December 2000. J. Dairy Sci. 85:295–350.
17. Kadzere C.T., Murphy M.R., Silanikove N., Maltz E. (2002). Heat stress in lactating dairy cows: a review. Livest. Prod. Sci., 77: 59–91.
18. Kamiya, M., Iwama, Y., Tanaka, M. and S. Shioya (2005) Effects of high ambient temperature and restricted feed intake on nitrogen utilization for milk production in lactating Holstein cows. Animal Science Journal 76(3): 217-223.
19. Kaylegian, K.E., R.C. Lindsay, 1995: In Handbook of milk fat Fractionation Technology and Applications AOCS Press, Champaign, III., 19-37
20. Knapp, D. M. & Grummer, R. R. Response of lactating dairy cows to fat supplementation during heat stress. J Dairy Sci 74, 2573–9 (1991).
21. Lacetera, N., Bernabucci, U., Ronchi, B. and A. Nardone (1996) Body condition score, metabolic status and milk production of early lactating dairy cows exposed to warm environment. Rivista di Agricoltura Tropicale e Subtropicale 90(1): 43-55.
22. Lacetera, N., Bernabucci, U., Ronchi, B. and A. Nardone (2003) Physiological and productive consequences of heat stress. The case of dairy ruminants. In: Lacetera, Bernabucci, Khalifa, Ronchi, Nardone (eds.) Interactions between climate and animal production. European Association Animal Production Technical Series No. 7, pp. 45-59.
23. Liu Z., V. Ezernieks, J. Wang, N. Wanni Arachchillage, J. B. Garner, W. J. Wales, B. G. Cocks & S. Rochfort, 2016. Heat Stress in Dairy Cattle Alters Lipid Composition of Milk. Scientific Reports | 7: 961 | DOI:10.1038/s41598-017-01120-9.
24. Mills, S., R.P.Ross, C.Hill, G.F.Fitzgerald, C.Stanton, 2011. Milk intelligence: Mining milk for bioactive substances associated with human health. International Dairy Journal (6): 377-401.
25. Muchenje, V., Dzama, K., Chimonyo, M., Strydom, P. E., Hugo, A., & Raats, J. G. (2009). Some biochemical aspects pertaining to beef eating quality and consumer health: A review. Food Chemistry, 112, 279–289.
26. Nantapo, C.T.W., V. Muchenje, A. Hugo, 2014. Atherogenicity index and health-related fatty acids in different stages of lactation from Friesian, Jersey and Friesian x Jersey cross cow milk under a pasture-based dairy system. Food Chemistry, 146: 127-133.
27. Nardone, A., Ronchi, B., Lacetera, N. and U. Bernabucci (2006) Climatic effects on productive traits in livestock. Veterinary Research Communications 30(Suppl.1): 75-81.
28. Parodi, P. W. Milk fat in human nutrition. Australian J. of Dairy Technol 59 (2004).
29. Purwanto B.P., Abo Y., Sakamoto R., Furumoto F., Yamamoto S. (1990). Diurnal patterns of heat production and heart rate under thermoneutral conditions in Holstein Friesian cows differing in milk production. J. Agric. Sci., 114: 139–142.
30. Renna Manuela, Carola Lussiana, Vanda Malfatto, Antonio Mimosi and Luca Maria Battaglini, 2010. Effect of exposure to heat stress conditions on milk yield and quality of dairy cows grazing on Alpine pasture. 9th European IFSA Symposium, 4-7 July 2010: 1338-1348.
31. Rhoads, M. L. et al. Effects of heat stress and plane of nutrition on lactating Holstein cows: I. Production, metabolism, and aspects of circulating somatotropin. J Dairy Sci 92, 1986–97 (2009).
32. Roman-Ponce, H., Thatcher, W. W., Buffington, D. E., Wilcox, C. J. & Van Horn, H. H. Physiological and Production Responses of Dairy Cattle to a Shade Structure in a Subtropical Environment. J Dairy Sci 60, 424–430 (1977).
33. Sevi, A., Annichiarico, G., Albenzio, M., Taibi, L., Muscio, A. and S. Dell'Aquila (2001) Effects of solar radiation and feeding time on behavior, immune response and production of lactating ewes under high ambient temperature. Journal of Dairy Science 84(3): 629-640.
34. Silanikove N. 2000. Effects of heat stress on the welfare of extensively managed domestic ruminants. Livestock Production Science, 67 , 1–18
35. Simopoulos, A. P., 2002. The importance of ratio of omega-6/omega-3 essential fatty acids. Biomedicine Pharmacotherapy, 56: 365-379.
36. Smith D. L., T. Smith, B. J. Rude, and S. H. Ward, 2013. Comparison of the effects of heat stress on milk and component yields and somatic cell score in Holstein and Jersey cows - Short communication. J. Dairy Sci. 96: 3028–3033
37. Srikandakumar, A., Johnson EH, 2004. Effect of heat stress on milk production, rectal temperature, respiratory rate and blood chemistry in Holstein, Jersey and Australian Milking Zebu cows. Trop Anim Health Prod. 2004 Oct;36(7):685-92.
38. West, J. W. Effects of heat-stress on production in dairy cattle. J Dairy Sci 86, 2131–44 (2003)
39. West, J. W. Effects of heat-stress on production in dairy cattle. J Dairy Sci 86, 2131–44 (2003).
40. Wheelock, J. B., Rhoads, R. P., Vanbaale, M. J., Sanders, S. R. & Baumgard, L. H. Effects of heat stress on energetic metabolism in lactating Holstein cows. J Dairy Sci 93, 644–55 (2010).
41. Zimbelman, R. B., Collier, R. J., Rhoads, R. P., Rhoads, M. L., Duff, G. C. & Baumgard, L. H. (2009). A re-evaluation of the impact of temperature humidity index (THI) and black globe humidity index (BGHI) on milk production in high-producing dairy cows. Proc. 24th Southwest Nutrition and Management Conf.

Received 16 June 2020
Accepted 17 July 2020

Investigation of *Mycoplasma* Species in Diseased and Healthy Calves and Heifers in Al-Najaf Province, Iraq

Khaleel Zainel Khaleel Al-Alo[1], Ayyed Bajee Hassan Alzwghaibi[2], Hassan Khalaf Ulaiwi Al-Karagoly[3], Salim Albukhaty[4]

[1]Department of Veterinary Clinical Sciences, Faculty of Veterinary Medicine, University of Kufa, Iraq, Najaf, Iraq;
[2]Faculty of Veterinary Medicine, Al Qasim Green University, Babylon, Iraq;
[3]Department of Internal and Preventive Medicine, College of Veterinary Medicine, University of Al-Qadisiyah, Diwaniyah, Iraq;
[4]Deptartament of Basic Sciences, University of Misan, Maysan, Iraq

Keywords: *Mycoplasma, ocular lesions, cattle, Iraq.*

Abstract. *Mycoplasma infections are among the most common causes of eye infection in cattle worldwide. The purpose of this work was to use a DNA-based method to investigate the presence of Mycoplasma spp. in the conjunctival sac of diseased and healthy calves and heifers. Between May 2018 and August 2019, a total of 116 eye swab samples were taken from seven industrial dairy farms in Al-Najaf province, where 85 samples were collected from diseased animals and 31 from healthy animals. Concerning the PCR test, 6.4% and 28.2% were respectively positive for healthy and diseased animals. Conjunctivitis, keratoconjunctivitis and corneal ulcers were reported in 52%, 31% and 17% of the diseased group, respectively. In conclusion, Mycoplasma spp. important bovine pathogen causing multiple eye lesions that can not be identifed due to their asymptomatic nature.*

Introduction

Corneal and conjunctival infections are amongst the most common eye diseases in livestock worldwide. A predisposing eye infection was considered to be eye irritation caused by exposure to sunlight, dust, pollen, seeds, and buds. Most cases of ocular lesions show corneal ulcers, corneal blindness, corneal oedema, light anxiety, blepharospasm, and abundance of tear excretion (Underwood et al., 2015, Brooks et al., 2017).

Mycoplasma is one of the main pathogens affecting a competitive animal health condition in most countries (Nicholas & Ayling, 2003). Mycoplasma exploits chronic diseases that affect the animal, leading to the suppression of the body's general immune status, providing an appropriate environment for infection with different *Mycoplasma* species. In cattle, various *Mycoplasma* species are considered to be a real cause of mastitis, respiratory tract infections, arthritis, genital tract infections, otitis, keratoconjunctivitis, and abortion (Maunsell & Donovan, 2009, Calcutt et al., 2018), which leads to massive economic losses in the sector of animals worldwide (Aebi et al., 2012, Matilda et al., 2018, Loria et al., 2018).

Moraxella bovis is indeed among the most common causes of eye diseases in cattle, but mycoplasma is no less harmful. Other pathogens like *Moraxella ovis* and *Chlamydia species* are also regarded as a common cause. Mycoplasma has many species, including *M. agalactiae, M. bovis*, *M. californicum, M. bovirhinis, M. alkalescens*, *M. mycoides*, *M. dispar*, *M. canadense*, *M. bovigenitalium, M. conjunctivae,* and *M. bovoculi* (Loria et al., 2018, Salih & Rosenbusch, 2001, Nicholas et al., 2000).

Mycoplasma alone can cause an ocular lesion in cattle, but a mixed infection with *Moraxella bovis* may increase the severity and pathogenicity of the disease (Alberti et al., 2006, Gould et al., 2013).

Mycoplasma is a self-reproducing microorganism that is recognized as the smallest one. They spread globally, either as free-living or as parasites of humans, animals and plants (Razin, 1992). Mycoplasmas are phenotypically distinct from other bacteria by their minute size and complete absence of a cell wall, as well as restricted metabolic ability (Razin et al., 1998). Using antibiotics such as penicillin that inhibit cell wall synthesis is futile in treating mycoplasma diseases because it lacks the cell wall (Klöckner et al., 2016, Naveed et al., 2020).

Because of restricted genetic potency of mycoplasmas, they need a complicated growth medium to consist of the medium enriched with peptone, yeast extract, animal serum and animal cell (Smith, 2012, Gerdtzen, 2017). There is a limited condition for mycoplasma growth, such as a specific host, a specific tissue and a specific organ, that reflects the precise nutritional character (Volokhov et al., 2011)

In order to control mycoplasma infections, a precise diagnosis must be made and suspected animals must be exluded from the herd because there is no effective vaccine against mycoplasma infections (Nicholas et al., 2008b, HA, 2013).

In addition to the shared antigenicity among pathogenic and non-pathogenic mycoplasmas, the costly and time-consuming culture of mycoplasmas make diagnosis difficult by routine technique (Pilo et al., 2007, Smith, 2012). The PCR test provides a more

Correspondence to Hassan Al-Karagoly, Department of Internal and Preventive Medicine, College of Veterinary Medicine, University of Al-Qadisiyah, Diwaniyah, Iraq
E-mail: hassan.aliwee@qu.edu.iq

precise method of mycoplasma detection in both acute and chronic infections and offers a greater scope for detection than culture and serological methods (Rossetti et al., 2010, Touati et al., 2014).

The occurrence of mycoplasma in Iraqi cattle is unclear, and not all diagnostic laboratories do isolate mycoplasma generally. For this reason, a DNA-based approach was employed to identify the presence of *Mycoplasma* spp. in the conjunctival sac of diseased and healthy calves and heifers.

2. Materials and Methods

2.1. Sample collection

A total of 116 eye swab specimens were collected from 85 diseased and 31 healthy animals during the study period between May 2018 and August 2019. The samples were taken from calves and heifers (6-2 months) of Holstein Friesian breed from 7 industrial dairy farms in Al-Najaf province-Iraq and transported by a cold chain to the laboratory. Clinical symptoms of the affected animals were reported. Furthermore, clinical cases were divided into three categories according to complication severity: conjunctivitis as a single sign; conjunctivitis and keratitis without corneal ulcers; conjunctivitis and keratitis involving a visible corneal ulcer.

The eye swab samples were taken from the conjunctival sac using sterile cotton moistened with sterile normal saline and stored in sterile tubes containing 1.5 mL of saline phosphate buffer and an ice pack for transfer to the laboratory to be stored at –70°C.

2.2. Preparing of DNA samples

DNA was extracted from the eye swab samples according to the protocol (Sinagen DNPTM kit) (SinaClonBioScience-Tehran, Iran).

2.3. PCR test

A 280 bp fragment of a highly conserved 16s rRNA coding region of mycoplasma genome was amplified using *Mycoplasma* spp. PCR detection Kit primer (SinaClonBioScience Company, Tehran, Iran). For this purpose, a final volume of 25 μL, consisting of 20 μL PCR MIX, 0.2μL Taq DNA polymerase and 4.8 μL DNA sample, was prepared in a PCR tube on ice. The 20 μL PCR MIX used in the reaction includes, in addition to $MgCl_2$ and dNTPs, specific primers for the 16s rRNA coding region. The PCR tubes containing the reaction mixture were then transferred to the thermocycler device (Bio-Rad™ - MJ Mini thermal cycler, USA). The thermocycling protocol was as follows: 1 cycle of 94°C for 2 min, 35 cycles of 94°C for 15 sec, 52°C for 20 sec and 72°C for 35 sec, with a terminal step of 5 min at 72°C. After completion of the thermocycler step, the PCR product samples were transferred to electrophoresis (5 volts/cm) using GelRed® Prestain Plus 6X DNA loading dye (Biotium, USA) with 1.5% agarose/TBE gels. Then the UV illuminator gel documentary (Siemens, Germany) was used to visualize a 280 bp amplicon. Positive and negative controls were also applied along with the samples (McAuliffe, Ellis, Ayling, & Nicholas, 2003; Tenk et al., 2006; Jain, Verma, & Pal, 2012).

Statistical analysis

Data obtained are expressed as mean ± SD. Using Excel and SPSS V.20 statistical applications, $P < 0.01$ values were considered statistically important.

Results

The PCR test of the current study indicated the presence of 280 bp fragments of 16s rRNA-specific coding gene of mycoplasma reflecting positive results, as shown in Fig. 1.

In general, 73.3% of the samples were taken from animals with different ocular lesions. The remainder of the 26.7% was collected without clinical evidence from healthy animals. Based on the findings of the polymerase chain reaction, overall positive samples were found to be 22.4% (26/116), 6.4% (2/31) in healthy animals, and 28.2% (24/85) in diseased animals (Fig. 2).

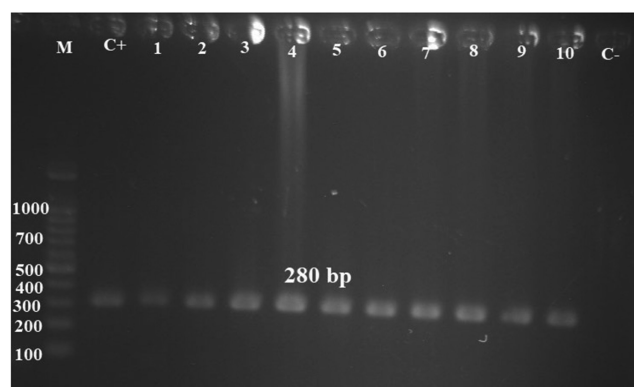

Fig. 1. Positive 280 bp bands of the Mycoplasma specified 16s rRNA gene coding region

Lane 1–10 shows eye swab samples. Lane C+ and Lane C– are both positive and negative controls. M: DNA ladder 100 bp.

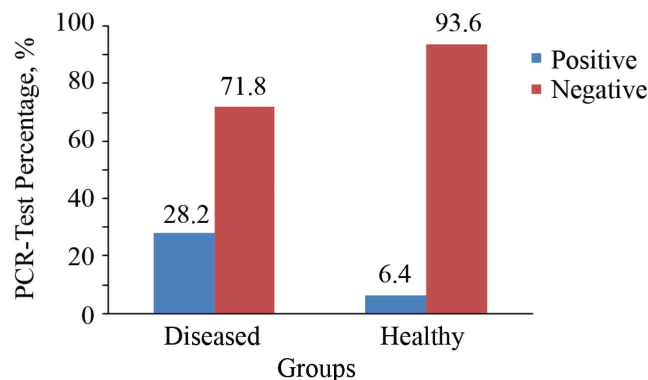

Fig. 2. Percentage of positive and negative results of the PRC test for both diseased and healthy animals

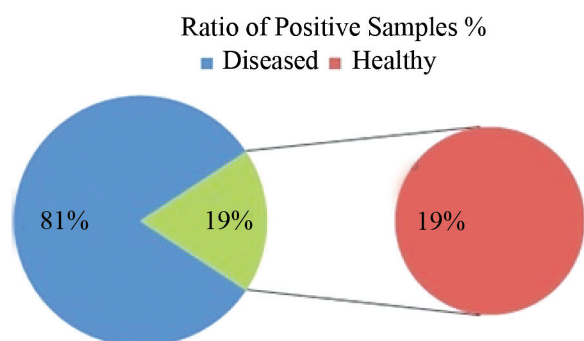

Fig. 3. The ratio of mycoplasm in healthy and diseased animals detected by PCR

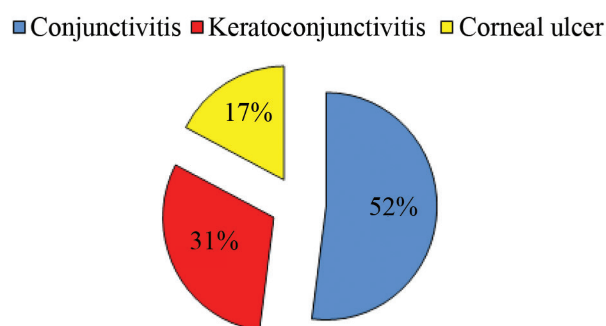

Fig. 4. Percentage of conjunctivitis, keratoconjunctivitis, and corneal ulcer in the positive samples of the diseased group

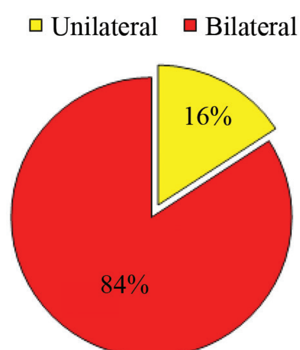

Fig. 5. Percentage of bilateral eye lesions

The ratio of identified microorganisms from diseased and healthy animals was 81% and 19%, respectively, revealing significant statistical differences (Fig. 3).

Conjunctivitis, keratoconjunctivitis and corneal ulcer were observed in 52%, 31% and 17% of the diseased animals, respectively, and 16% of the cases showed involvement of both eyes (bilateral infection) (Figs. 4 and 5).

Discussion

M. bovoculi and *M. conjunctivae* are the two most clinically important species to cause conjunctivitis and infectious bovine keratitis (IKC) (Romano et al., 2018, Tryland et al., 2017, Gupta et al., 2015).

In this study, a polymerase chain reaction was performed to determine the *Mycoplasma* species in the conjunctival sac of diseased and healthy calves and heifers.

In the current study, a 280 bp 16s rRNA fragment of *Mycoplasma* spp. was specifically detected. Besides, cross-amplification did not occur with other pathogens. The *Mycoplasma* spp. DNA sequence analysis (the data not shown) confirmed the positive result of the PCR test.

The total percentage of *Mycoplasma* spp. in eye swab samples was 22.4% (26/116). This percentage is lower than the proportion recorded by (Gupta et al., 2015) and (Raoofi et al., 2016) who reported a ratio of 37.5% and 56.7%, respectively. The difference can relate to the number of samples included in the study, the study season, the age of the animals studied and the area. In the diseased animals, the positive percentage of Mycoplasma identified was 28.2, while in healthy animals it was 6.4. We disagree with the results (Schöttker-Wegner et al., 1990) of 34.2% and 41.2%, respectively, in ill and healthy cattle, as well as the findings of Raoofi et al., (2016) who revealed 63.8% and 48.1% in infected and non-infected cattles. That can be attributed to the difference in both the total number of samples being examined and the season.

The present study shows that conjunctivitis, keratoconjunctivitis and corneal ulcers were found in 52%, 31% and 17% of the diseased group, respectively. Such findings are in line with those reported by (Sidal et al., 2007) who reported a ratio of 30.3%, 33.3%, and 36.4%, respectively. Infections of both eyes were detected in 16% of the cases, and this result is consistent with that of (Takele & Zerihun, 2000). Variation in virulence of different *Mycoplasma* spp., heat, dust, host immune status and likely concurrent infection with a bovine rhinotracheitis virus (bovine herpesvirus 1) or *Moraxella bovis* increases disease severity (Atkinson et al., 2008, Maunsell et al., 2011, Wilcock & Njaa, 2016).

Severe conjunctivitis with corneal opacity or ulceration is the most frequent and obvious sign of Mycoplasma ocular infection. Eyelid involvement with marked swelling is prominent. Conjunctivitis is prominent in many keratoconjunctivitis-producing infections (Schnee et al., 2015).

Corneal ulcers initially arise from the cytotoxic effect of the microorganism over 24 hours, resulting in corneal degradation (microscopically) without a sufficient inflammatory response (Nicholas et al., 2008a, Brooks, 2005). The corneal epithelium is eventually destroyed, followed by degradation of the keratocytes and degradation of the corneal stroma. An inflammatory response will then develop for several days leading to the development of large and deep corneal ulcers and oedema with stromal involvement, as well as corneal neovascularization (CNV) (Maggs, 2008).

Conclusion

The study found that the percentage of *Mycoplasma* spp. in animals with eye lesions was greater than in healthy animals, but only showing its existence in healthy animals suggests the latent or asymptomatic nature. Hence, *Mycoplasma* spp. infection can contribute to the severity of disease-related lesions.

Conflicts of Interest

The authors guarantee that the substances discussed at some stage in this manuscript are not affiliated with any business or entity having a financial interest or non-financial interest.

Acknowledgments

All thanks and appreciation to the workers at the cow breeding stations and industrial dairy farms in Al-Najaf province, Iraq, for facilitating the sampling collection process

References

1. Underwood WJ, Blauwiekel R, Delano ML, Gillesby R, Mischler SA, and Schoell A. Biology and diseases of ruminants (sheep, goats, and cattle). Laboratory animal medicine: Elsevier2015. P.623-94.
2. Nicholas R, and Ayling R. Mycoplasma bovis: disease, diagnosis, and control. Research in veterinary science 2003.74(2), P. 105-12
3. Maunsell FP, and Donovan GA. Mycoplasma bovis infections in young calves. Veterinary Clinics of North America: Food Animal Practice 2009.25(1), P. 139-77
4. Aebi M, Bodmer M, Frey J, and Pilo P. Herd-specific strains of Mycoplasma bovis in outbreaks of mycoplasmal mastitis and pneumonia. Veterinary microbiology 2012. 157(3-4), P. 363-68
5. Alberti A, Addis MF, Chessa B, Cubeddu T, Profiti M, Rosati S, Ruiu A, and Pittau M. Molecular and antigenic characterization of a Mycoplasma bovis strain causing an outbreak of infectious keratoconjunctivitis. Journal of Veterinary Diagnostic Investigation 2006. 18(1), P. 41-51
6. Atkinson TP, Balish MF, and Waites KB. Epidemiology, clinical manifestations, pathogenesis and laboratory detection of Mycoplasma pneumoniae infections. FEMS microbiology reviews 2008. 32(6), P. 956-73
7. Ayling R, Bashiruddin S, and Nicholas R. Mycoplasma species and related organisms isolated from ruminants in Britain between 1990 and 2000. Veterinary Record 2004. 155(14), P. 413-16
8. Björnelius E, Jensen JS, and Lidbrink P. Conjunctivitis associated with Mycoplasma genitalium infection. Clinical Infectious Diseases 2004. 39(7), P. e67-e69
9. Brooks DE. Equine stromal and endothelial keratopathies: medical management of stromal abscesses, eosinophilic keratitis, calcific band keratopathy, striate band opacities, and endotheliitis in the horse. Clinical Techniques in Equine Practice 2005. 4(1), P. 21-28
10. Brooks DE, Matthews A, and Clode AB. Diseases of the cornea. Equine ophthalmology 2017.3,P. 252-368
11. Calcutt M, Lysnyansky I, Sachse K, Fox L, Nicholas R, and Ayling R. Gap analysis of Mycoplasma bovis disease, diagnosis and control: an aid to identify future development requirements. Transboundary and emerging diseases 2018. 65, P. 91-109
12. Gerdtzen ZP. Medium Design, Culture Management, and the PAT Initiative. Applied Bioengineering 2017. P. 2:8
13. Gould S, Dewell R, Tofflemire K, Whitley RD, Millman ST, Opriessnig T, Rosenbusch R, Trujillo J, and O'Connor AM. Randomized blinded challenge study to assess association between Moraxella bovoculi and infectious bovine keratoconjunctivitis in dairy calves. Veterinary microbiology 2013. 164(1-2), P. 108-15
14. Gupta S, Chahota R, Bhardwaj B, Malik P, Verma S, and Sharma M. Identification of chlamydiae and mycoplasma species in ruminants with ocular infections. Letters in applied microbiology 2015. 60(2), P. 135-39
15. Kharel R, Poddar C,and Biswas J. Pathology of Endophthalmitis. Endophthalmitis: Springer 2018. P. 333-41.
16. Klöckner A, Bühl H, Viollier P, and Henrichfreise B. Deconstructing the chlamydial cell wall. Biology of Chlamydia: Springer, 2016. P. 1-33.
17. Loria G, Puleio R, and Nicholas R. Contagious Agalactia: economic losses and good practice. J Bacteriol Mycol 2018. 5(5), P. 1076
18. Maggs DJ. CORNEA AND SCLERA 10. Slatter's fundamentals of veterinary ophthalmology 2008. P. 175
19. Matilda A-A, Linda AA, Cornelius A, Korley KN, and Paa TA. Activity of Mycoplasma synoviae in Commercial Chickens in Ghana: Serological Evidence. Advances in Life Science and Technology 2018.62
20. Maunsell F, Woolums A, Francoz D, Rosenbusch RF, Step DL, Wilson DJ, and Janzen ED. Mycoplasma bovis infections in cattle. Journal of Veterinary Internal Medicine 2011. 25(4), P. 772-83
21. Naveed M, Chaudhry Z, Bukhari SA, Meer B, and Ashraf H. Antibiotics resistance mechanism. Antibiotics and Antimicrobial Resistance Genes in the Environment: Elsevier, 2020:292-312.
22. Nicholas R, Ayling R, and Loria G. Ovine mycoplasmal infections. Small Ruminant Research 2008. 76(1-2), P. 92-98
23. Nicholas R, Ayling R, and McAuliffe L. *Mycoplasma diseases of ruminants*: CABI 2008.
24. Nicholas R, Baker S, Ayling R, and Stipkovits L. Mycoplasma infections in growing cattle. Cattle Practice 2000. 8(2), P. 115-18
25. Pilo P, Frey J, and Vilei EM. Molecular mechanisms of pathogenicity of Mycoplasma mycoides subsp. mycoides SC. The Veterinary Journal 2007. 174(3), P. 513-21
26. Pup HA, and Lindley EP. Diseases of Cattle in the Tropics: Economic and Zoonotic Relevance 2013;6:255
27. Raoofi A, Mottaghian P, Madadgar O, and Badiei A. A Survey on The Prevalence of Mycoplasmal Infections in Infectious Bovine Keratoconjuntivitis 2016
28. Razin S. 1. Mycoplasma taxonomy and ecology. AMERICAN SOCIETY FOR MICROBIOLOGY, WASHINGTON, DC(USA). 1992. P. 3-22
29. Razin S, Yogev D, and Naot Y. Molecular biology and pathogenicity of mycoplasmas. Microbiology and molecular biology reviews 1998. 62(4), P. 1094-156
30. Romano JS, Mørk T, Laaksonen S, Romano JS, Torill M, Sauli L, Erik Å, Ingebjørg H, Nymo, Marianne S, and Morten T. Infectious keratoconjunctivitis in semi-domesticated Eurasian tundra reindeer (Rangifer tarandus tarandus): microbiological study of clinically affected and unaffected animals with special reference to cervid herpesvirus 2. BMC veterinary research 2018. 14(1), P15
31. Rossetti BC, Frey J, and Pilo P. Direct detection of Mycoplasma bovis in milk and tissue samples by real-time PCR. Molecular and cellular probes 2010. 24(5), P. 321-23
32. Salih B, and Rosenbusch R. Cross-reactive proteins among eight bovine mycoplasmas detected by monoclonal antibodies. Comparative immunology, microbiology and infectious diseases 2001. 24(2), P. 103-11
33. Schnee C, Heller M, Schubert E, and Sachse K. Point prevalence of infection with Mycoplasma bovoculi and Moraxella spp. in cattle at different stages of infectious bovine keratoconjunctivitis. The Veterinary Journal 2015. 203(1), P. 92-96
34. Schöttker-Wegner H, Binder A, and Kirchhoff H. Detection of mycoplasmas in eye swab specimens of cattle. Zentralblatt

fur Veterinarmedizin. Reihe B. Journal of veterinary medicine. Series B 1990. 37(6), P. 436-41
35. Sidal M, Kilic A, Unuvar E, Fatma O, Mustafa O, Ali A, Derya A, Kaya K, and Hayati B. Frequency of Clamydia pneumoniae and Mycoplasma pneumoniae Infections in Children. Journal of tropical pediatrics 2007. 53(4), P. 225-31
36. Smith PF. *The biology of mycoplasmas*: Elsevier 2012.
37. Touati A, Peuchant O, Jensen JS, Bébéar C, and Pereyre S. Direct detection of macrolide resistance in Mycoplasma genitalium isolates from clinical specimens from France by use of real-time PCR and melting curve analysis. Journal of clinical microbiology 2014. 52(5), P. 1549-55
38. Tryland M, Romano JS, Marcin N, Ingebjørg HN, Terje DJ, Karen KS, and Torill M. Cervid herpesvirus 2 and not Moraxella bovoculi caused keratoconjunctivitis in experimentally inoculated semi-domesticated Eurasian tundra reindeer. Acta Veterinaria Scandinavica 2017. 59(1), P. 23
39. Takele G, and Zerihun A. Epidemiology of Infectious Keratoconjunctivitis in Cattle in South-east Ethiopia. Journal of Veterinary Medicine Series A 2000. 47(3), P. 169-73
40. Volokhov DV, Graham LJ, Brorson KA, and Chizhikov VE. Mycoplasma testing of cell substrates and biologics: Review of alternative non-microbiological techniques. Molecular and cellular probes 2011. 25(2-3), P. 69-77.

Received 28 June 2020
Accepted 25 July 2020