UDC636

Print ISSN 1450-9156
Online ISSN 2217-7140

BIOTECHNOLOGY IN ANIMAL HUSBANDRY

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VOL 37, 1
Founder and publisher
INSTITUTE FOR ANIMAL HUSBANDRY
11080 Belgrade-Zemun
Belgrade 2021

Journal for the Improvement of Animal Husbandry

UDC636

Print ISSN 1450-9156
Online ISSN 2217-7140

BIOTECHNOLOGY IN ANIMAL HUSBANDRY

Belgrade - Zemun 2021

Biotechnology in Animal Husbandry 37, (1), 1-73. 2021 ISSN 1450-9156
Publisher: Institute for Animal Husbandry, Belgrade-Zemun UDC 636

Address of the Editor's office
Institute for Animal Husbandry, Autoput 16, P. Box 23, 11080 Belgrade-Zemun, Republic of Serbia Tel. 381 11 2691 611, 2670 121; Fax 381 11 2670 164;
e-mail: biotechnology.izs@gmail.com; www.istocar.bg.ac.rs

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Annual subscription: for individuals -500 RSD, for organizations 1200 RSD, - foreign subscriptions 20 EUR. Bank account Institut za stočarstvo, Beograd-Zemun 105-1073-11 Aik banka Niš Filijala Beograd.

Journal is published in four issues annually, circulation 100 copies.

The publication of this journal is sponsored by the Ministry of Education and Science of the Republic of Serbia.
Printed: "Goragraf", Ul. Živka Petrovića 11 Zemun,

Biotechnology in Animal Husbandry 37 (1), 1-15, 2021
Publisher: Institute for Animal Husbandry, Belgrade-Zemun
ISSN 1450-9156
UDC 619:616'62
https://doi.org/10.2298/BAH2101001N

LABORATORY DIAGNOSTICS OF BOVINE PARAINFLUENZA-3 VIRUS, BOVINE HERPESVIRUS 1, AND BOVINE RESPIRATORY SYNCYTIAL VIRUS ASSOCIATED WITH BOVINE RESPIRATORY DISEASE

Jakov Nišavić[1], Nenad Milić[1], Andrea Radalj[1], Aleksandar Stanojković[2], Ljubiša Veljović[3]

[1] Department for Microbiology, Faculty of Veterinary Medicine, University of Belgrade, 11000 Belgrade, Serbia
[2] Institute for Animal Husbandry, Belgrade – Zemun, 11080 Zemun, Serbia
[3] Scientific Veterinary Institute of Serbia, 11000, Belgrade, Serbia
Corresponding author: Andrea Radalj, andrea.zoric@vet.bg.ac.rs
Review paper

Abstract: The bovine respiratory disease complex (BRDC) is multifactorial and results from interactions between host factors, environmental factors, and pathogens. A virus, as an initial pathogen alters the animal's immunity supporting the bacterial colonization of the lower respiratory tract. Bovine herpesvirus 1 (BHV-1), bovine parainfluenza virus 3 (BPIV-3), and bovine respiratory syncytial virus (BRSV) are among the most significant viruses associated with BRDC. The disease most often affects young and older immunosuppressed animals. Laboratory results depend on the selected sampling site of the respiratory tract and proper timing during the period of virus shedding. The samples for testing mostly include nasal or nasopharyngeal swabs, tracheal wash, bronchoalveolar lavage fluid, or necropsy specimens. Virus isolation, although considered as the gold standard, is time-consuming and depends on the virus species and sampling conditions. Most of the virus identification methods used today are molecular assays (conventional and real-time PCR or RT-PCR) that are rapid, sensitive, and specific, which is of the essence in veterinary diagnostic laboratories. DNA sequencing is mostly used to detect specific genetic mutations and for molecular epidemiology of disease outbreaks. Serological diagnosis is performed based on the detection of specific antibody presence after infection of seronegative animals or a 4-fold specific antibody titer rise in paired serum samples. Different assays are available, including virus neutralization, complement fixation, haemagglutination inhibition, and ELISA. The early and reliable diagnosis is beneficial in the management and control of BRDC and is the basis of a timely treatment and prevention program.

Key words: BRDC, BHV-1, BPIV-3, BRSV, laboratory diagnosis

Introduction

Respiratory diseases lead to significant economic losses in cattle production and cause increased morbidity and mortality, with negative long-term consequences for herd health and productivity, including reproductive disorders, reduced milk production, and shortened lifespan. The disease prevalence increases in a stressful, overcrowded environment, and suboptimal environmental conditions (*Ellis, 2001; Van Der Fels-Klerx et al., 2002*). The bovine respiratory disease complex (BRDC) is multifactorial and is a consequence of different interactions between host factors, environmental factors, and pathogens. An initial pathogen alters the animal's immune mechanisms, allowing colonization of the lower respiratory tract by bacteria (*Ellis, 2009*). The BRDC can be caused by one or several primary pathogens including *Mycoplasma* species and respiratory viruses, followed by a secondary bacterial infection, or in some cases by bacteria unassisted. Some of the most common viral agents include the bovine respiratory syncytial virus (BRSV), bovine parainfluenza-3 virus (BPIV-3), and bovine herpesvirus 1 (BHV-1). In some cases, viruses can cause the appearance of clinical symptoms without bacterial superinfection, still, their role in the development of the clinical picture of respiratory diseases in cattle is mainly cofactorial (*Van Der Fels-Klerx et al., 2002; Ellis, 2009; Oliveira et al., 2020*). The bovine respiratory syndrome is a disease that most often affects young animals as well as older immunosuppressed individuals (*Ellis, 2001; Gershwin, 2012; von Messling, 2016*). The interpretation of laboratory test results considerably depends on the selected sampling site of the respiratory tract. Nasal swabs are frequently used as this sampling method is quick and uncomplicated, especially in large cattle populations (*Veljović et al., 2016; Nišavić et al., 2018a*). However, these are may not be applicable, especially for pathogens that replicate in the lower parts of the respiratory tract (*Ellis, 2009; von Messling, 2016; Kamdi et al., 2020*). Moreover, proper timing is of the essence and sampling should be performed during the period of virus shedding (*Grissett et al., 2015*). Nasopharyngeal swabs are usually more relevant, though the retrieved samples often contain many different microorganisms, sometimes affecting proper diagnosis. On the other hand, bronchoalveolar lavage is performed by endoscopy and can be meaningful in specific situations, e.g. when targetting the affected lung lobe (*Oliveira et al., 2020; Pardon and Buczinski, 2020*). A variety of laboratory tests can be used to identify respiratory viruses in field specimens collected during respiratory outbreaks in cattle. Virus isolation in cell culture and subsequent identification by neutralizing antibodies or immunofluorescence is often arduous and time

consuming for veterinary diagnostic laboratories, especially when timely diagnosis is of the essence (*Valarcher and Taylor, 2007; Milić et al., 2010; von Messling, 2016; Leme et al., 2020*). Serologic methods such as the virus neutralization (VN) test, complement fixation test (CFT), enzyme-linked immunosorbent assay (ELISA), etc. are useful to evaluate infection dynamics or to determine the protective status of vaccination. However, these methods are not appropriate when the objective is to implement adequate therapy (*Šamanc et al., 2009; Sibhat et al., 2018; Pardon and Buczinski, 2020*). Today, most of the methods for virus identification rely on molecular assays based on polymerase chain reaction (conventional and real-time PCR or RT-PCR), thus adding to the speed, sensitivity, and specificity of virological diagnostics (*Boxus et al., 2005; Brodersen, 2010;Veljović et al., 2016; OIE, 2018*). The methods of DNA sequencing are mostly used for the detailed examination of the identified virus strains such as the detection of specific genetic mutations and molecular epidemiology of disease outbreaks, i.e. tracing the infection source and viral strain distribution (*Veljović et al., 2016; Krešić et al., 2018; Nišavić et al., 2018a; Nišavić et al. 2018b, Leme et al., 2020*).

Bovine parainfleunza 3 virus (BPIV-3)

Bovine parainfluenza-3 virus (BPIV-3) belongs to the genus *Respirovirus* and the family *Paramyxoviridae*. The viral genome consists of negatively oriented, unsegmented, and single-stranded RNA encoding six structural proteins: nucleocapsid protein, phosphoprotein, matrix (M) protein, fusion (F) protein, hemagglutinin-neuraminidase (HN) protein, and large (L) polymerase protein. The HN protein of the outer viral envelope is crucial for binding to cell surface receptors, while the F protein allows the fusion to the cell membrane, followed by the entry of the nucleocapsid into the host cell. Protein M interacts with surface glycoproteins HN and F and directs their insertion and aggregation at specific sites in the cell membrane (*Nišavić et al., 2006a; Nišavić et al., 2006b*; *Ellis, 2010; Veljović et al., 2016; Sobhy et al., 2017; ICTV, 2020*). This virus causes respiratory disease in cattle, and the clinical symptoms are manifested by anorexia, cough, fever, shortness of breath, and diarrhea in some cases. The bovine parainfluenza-3 virus has an immunosuppressive effect and causes bronchial pneumonia, which often occurs as a consequence of the primary viral and secondary bacterial infection. As previously stated, nonspecific environmental factors are a prerequisite for disease development (*Ellis, 2001; Ellis, 2010; Sobhy et al., 2017*). To date, three genotypes of parainfluenza-3 virus have been described, namely A, B and C. Genotype A was first described in the United States, and genotypes B and C in Australia and China (*Zhu et al., 2011; Oem et al., 2013*). The identification of new BPIV-3 genotypes is significant in view of the improvement of diagnostic methods and vaccine production (*Oem et al., 2013*).

Diagnosis of a BPIV-3 infection is based on the detection of live virus, viral antigen, or viral nucleic acid directly from animal samples or on the increase in specific antibody titers in paired serum samples. Samples for testing include nasopharyngeal swabs, tracheal wash, bronchoalveolar lavage fluid, or necropsy specimens (*Veljovic et al., 2016; von Messling, 2016; Oliveira et al., 2020*). The bovine parainfluenza-3 virus can be successfully isolated in cell lines of bovine origin such as bovine turbinate cells, or Madin-Darby bovine kidney cells (MDBK) (*Ellis, 2010; Veljović et al., 2016; Sobhy et al., 2017*). Identification of the virus in inoculated cell lines is based on the appearance of cytopathic effect (CPE), hemadsorption, or immunofluorescence staining (*Ellis, 2010; von Messling, 2016*). Viral RNA can be detected in suspected samples by RT-PCR or real-time RT-PCR (*Veljović et al., 2016; von Messling, 2016; Oliveira et al., 2020*). Usually, the BPIV3 shedding lasts for 1 to 2 days, the shedding peak occurs at day 4, and it mostly ceases in the first 10 to 13 days after infection (*Grissett et al., 2015*). In most cases, BPIV-3 is only detected during the first days after infection and is often absent when specimens are taken during secondary bacterial infections. Therefore, the detection of specific anti-BPIV-3 antibodies in paired serum samples can be performed by complement fixation, haemagglutination inhibition (HI), virus neutralization, or ELISA (*Ellis, 2010; von Messling, 2016*). The positive results of virological diagnostic tests should always be reviewed considering the high incidence of subclinical BPIV-3 infections and the multifactorial etiology of BRDC. Accordingly, the clinical condition of the herd and the sampled animal should always be assessed in parallel with laboratory results (*von Messling, 2016*). *Sobhy et al.* (*2017*) collected nasal swabs from 12 calves with clinical symptoms of pneumonia in Egypt and examined the presence of BPIV-3, BVDV, BRSV, and BHV-1 using the virus isolation method in MDBK cell line and the RT-PCR method. The presence of BPIV-3 was determined in eight samples, while the obtained results of the phylogenetic analysis showed that the Egyptian strains belong to genotype A. Additionally, the authors confirmed a higher prevalence of BPIV-3 in cattle compared to buffaloes. *Oliveira et al.* (*2020*) collected and examined 21 samples of bronchoalveolar lavage from 15 cows with clinical symptoms of respiratory infection, and 6 samples originating from asymptomatic cows. In 85.7% of the examined samples, the authors confirmed the presence of at least one causative agent, mixed infection was found in 72.2% of samples, while individual infection caused by only one pathogen was found in 27.7% of samples. Still, the presence of BHV-1, BPIV-3, and the bacterium *Mannheimia haemolytica* was not detected. In the studies of *Veljović et al.* (*2016*) performed in Serbia, 119 samples of nasal swabs were collected from cattle with clinical symptoms of respiratory infection and tested for the presence of BPIV-3 using virus isolation in the MDBK cell line and the RT-PCR method. The virus was successfully isolated from eight examined samples, and the identification was performed by the virus-neutralization test. All BPIV-3 strains detected in Serbia were assigned to genotype

C. Furthermore, the Serbian isolates showed a high degree of similarity with Chinese SD0805, SD0809, and SD0835 isolates, followed by isolates originating from South Korea, Japan, and the USA (TVMDL16 and TVMDL20). Concurrently, the Serbian strains differed from the BPIV-3 genotype B strains originating from Australia and the USA (TVMDL15 and TVMDL17). *Veljović et al.* (*2014*) examined 20 samples of bovine nasal swabs for the presence of BPIV-3 using virus isolation in the MDBK cell line and the RT-PCR method with specific F gene primers. The virus was identified in 4 samples, while F sequencing results demonstrated a high degree of similarity with analogous BPIV-3 sequences isolated in China and South Korea. Similarly, *Oem et al.* (*2013*) collected bovine nasal swabs from diseased animals with respiratory symptoms in South Korea. A total of five samples of bovine nasal swabs were examined using virus isolation, RT-PCR, and electron microscopy. The newly identified BPIV-3 strain differed from viruses belonging to genotypes A and B and was closely related to the Chinese SD0835 virus strain. Furthermore, the phylogenetic analysis revealed that the South Korean isolates belong to the C genotype indicating a correlation between the genetic variations in the viral genome and the geographical localization of isolates. In their tests, *Šamanc et al.* (*2009*) collected blood serum samples from unvaccinated cattle from nine farms in Serbia. A total of 92 samples were examined for the presence of antibodies against BRSV, BPIV-3, and BHV-1 using iELISA. Their results confirmed the presence of antibodies against BPIV-3, BRSV, and BHV-1 in 83.69%, 50%, and 20.65% of the samples, respectively.

Bovine herpesvirus 1 (BHV-1)

Bovine herpesvirus 1 (BHV-1) belongs to the genus *Varicellovirus*, the subfamily *Alphaherpesvirina*e, and the *Herpesviridae* family. It is a double-stranded DNA virus with an outer envelope containing glycoprotein antigens crucial for viral binding to cell surface receptors and the process of viral replication (*Osterrieder, 2016; ICTV, 2020*). BHV-1 causes infectious bovine rhinotracheitis (IBR), manifested by upper respiratory tract disease, conjunctivitis, and bronchopneumonia. The appearance of erosions and ulcers in the upper respiratory tract, mostly the trachea, are characteristic of BHV-1 infection (*Ellis, 2009*). Another infection form caused by this virus is known as infectious pustular vulvovaginitis, manifesting as inflammation of the vulva and vagina, balanoposthitis, and abortion (*Muylkens et al., 2007*). After transmission which usually occurs by direct contact, BHV-1 infects the epithelial cells of the upper airways, as well as nerves (*Ellis, 2009*). Like other herpesviruses, BHV-1 causes a lifelong latent infection with periodic virus reactivation from the neural tissue and shedding caused by stress and immunosuppression. Bovine herpesvirus 1 can trigger BRDC through immunosuppression that enables secondary infections leading to pneumonia and sometimes death (*Muylkens et al. 2007; Ellis, 2009;*

Osterrieder, 2016; Jones, 2020). This virus causes lysis of the ciliated epithelium of the trachea which contributes to the aggregation of bacteria in the upper airways, thereby resulting in pneumonia. Moreover, BHV-1 downregulates type 1 interferon, induces the apoptosis of CD4+ T cells, and reduces MHC I expression (*Ellis, 2009*). The average time for BHV-1 shedding is 2 days, while peak shedding occurs on day 4 post infection (*Grissett et al., 2015*).

This infection is mostly diagnosed by virus isolation in cell culture, serology, and PCR. BHV-1 can be isolated from nasal swabs, conjunctival swabs, tonsils, and lungs using cells of bovine origin or Vero cells, characterized by the appearance of rapid cytopathic effect with the formation of syncytia (*Nišavić et al., 2010; Biswas et al., 2013; Osterrieder, 2016*). The virus is further identified by virus neutralization test using BHV-1 antiserum or by direct detection of BHV-1 antigen by immunofluorescence (*OIE, 2018*). Specific anti-BHV-1 antibodies are detected in the serum of cattle within 2–3 weeks of infection (Graham et al., 1997; Biswas et al., 2013). Usually, the BHV-1 seroconversion takes place around day 18, reaching the peak on day 40 after infection (*Grissett et al., 2015*). The indirect and blocking ELISA are used more extensively than the virus neutralization test due to its suitability for screening large numbers of samples in a shorter period. It is usually necessary to test paired serum samples collected from animals suspected of BHV-1 infection (*Graham et al., 1997; OIE, 2018; Sibhat et al., 2018*). The polymerase chain reaction, especially the real-time PCR, is now routinely used for the detection of viral DNA in examined samples as a rapid and reliable method (*Milić et al., 2010; Biswas et al., 2013; OIE, 2018; Nišavić et al., 2018a; Oliveira et al., 2020*).

Milić et al. (*2010*) collected 65 samples of nasal swabs of calves and heifers from several farms in the Republic of Serbia. The samples were examined for the presence of BHV-1 using virus isolation and PCR with primers for thymidine kinase and glycoprotein B (gB) and real-time PCR with gB specific primers. Virus isolation in cell culture was unsuccessful, however, the use of conventional PCR and real-time PCR yielded 1 and 3 positive samples, respectively, justifying the use of molecular methods in the diagnostics of BHV-1 infection. Furthermore, 20 bovine nasal swab samples were examined for the presence of BHV-1 using the standard method of virus isolation and PCR, and accordingly, the virus was detected solely by molecular methods (*Nišavić et al., 2010*). Moreover, *Nišavić et al.* (*2018a*) collected 110 nasal swabs and determined the presence of BHV-1 in 4 samples by both virus isolation and PCR. Phylogenetic analysis of the gB gene of these Serbian isolates showed 100% similarity with analog BHV-1 sequences from Egypt and the USA, and 99% to 98% with BHV-1 strains from Israel, India, Brazil, and the USA. The analysis of the thymidine kinase encoding gene grouped Serbian BHV-1 strains with virus isolates from the USA and Australia. Multiplex PCR has proved to be a reliable and convenient method for simultaneous detection and differentiation between BHV-1 and BHV-5

using specific primers for glycoprotein C (gC) (*Claus et al., 2005*). Bovine herpesvirus 1 is divided into two genotypes, namely 1.1 and 1.2, and genotype 1.2 is further separated into BHV-1.2a and 1.2b. *Zhou et al.* (*2020*) examined 102 lung samples originating from calves that died with symptoms of respiratory infection. Samples were collected from animals originating from 13 different bovine populations in China. BHV-1 was isolated, and the selected isolates were phylogenetically analyzed using gC primers. The obtained results showed that all isolates belong to the BHV-1.2b gene subtype, which appears to be dominant in China. The molecular analysis of the gC encoding region of bovine herpesvirus isolates originating from Brazil, Uruguay, and Argentina was performed (*Traesel et al., 2013*). This study showed that the gene sequence encoding the viral glycoprotein C is suitable for phylogenetic analysis of BHV-1 and BHV-5 virus strains and confirmed a clear difference between BHV-1 and BHV-5 viruses as well as between BHV-1 virus subtypes (BHV-1.1 and BHV-1.2). BHV-1 infection monitoring programs are based on the detection of the presence of the virus and the differentiation between vaccinated and infected animals. Vaccination of cattle is performed with a vaccine virus strain devoid of glycoprotein E. Accordingly, *Wernike et al.* (*2011*) developed a highly sensitive triplex real-time PCR method that enables the differentiation of field and vaccine BHV-1 strains. The BHV-1 seroprevalence was examined recently in 1,379 dairy cows from 149 cattle populations in Ethiopia (*Sibhat et al., 2018*). The collected milk samples were tested using ELISA, which proved to be a convenient method, and all the cattle populations had an average level of BHV-1 seroprevalence of 81.8%.

Bovine respiratory synctytial virus (BRSV)

Bovine respiratory syncytial virus (BRSV) belongs to the genus *Orthopneumovirus* which belongs to the *Pneumoviridae* family. The virus possesses a single-stranded 15 kb RNA molecule and has an irregular virion shape ranging from almost spherical to filamentous. The viral envelope contains three significant glycoproteins: glycoprotein G that enables the binding to the host cell surface, fusion protein (F), and small hydrophobic (SH) protein (*von Messling, 2016; ICTV, 2020*). The fusion protein leads to the fusion of cell membranes with the consequent formation of syncytia, thus facilitating the virus movement from cell to cell. These three glycoproteins are responsible for protective immunity development (*Valarcher and Taylor, 2007; Gershwin, 2012*). The genetic variations of up to 11%, mostly in the G protein are a consequence of viral RNA-dependent RNA polymerase and the absence of exonuclease proofreading (*Larsen et al., 2000*). The BRSV G protein is often used in studies concerning the classification of this virus considering its genetic and antigenic heterogeneity (*Valarcher et al., 2000*). The bovine respiratory syncytial virus has been classified into four antigenic subgroups, A, B, AB, and untyped based on their G protein

antigenic differences, however, this association has unknown biological implications (*Valarcher and Taylor, 2007*). Based on the genetic differences od G and F encoding sequences, BRSV strains are currently divided into subgroups I to VIII (*Leme et al., 2020*). Infection caused by BRSV occurs in all age groups, but the disease most often affects recently weaned calves and young cattle, causing pneumonia and predisposing them to other respiratory infections. Clinical symptoms include fever, anorexia, depression, dyspnoea, mucous nasal discharge, while the so-called 'air-hunger' position of mouth breathing with the head and neck outstretched is also present in severe cases (*Gershwin, 2012; von Messling, 2016; Leme et al., 2020*). Cattle are the natural hosts of BRSV, however, it is possible that small ruminants also play a role in virus transmission (*von Messling, 2016*). This virus is mainly transmitted through direct contact or by aerosol and it replicates in the superficial layer of the respiratory ciliated epithelium and replication can also be detected in type II pneumocytes (*Valarcher and Taylor, 2007; von Messling, 2016*). Bovine respiratory syncytial virus infection contributes to the development of secondary bronchopneumonia since bacteria deposit in the lower respiratory tract as a result of the damage to the mucociliary escalator function (*Ellis, 2009*). The shedding of BRSV can usually be detected on day 3 postinfection with the average time to peak on day 5, and time to resolution between days 7–14 (*Grissett et al., 2015*).

The diagnosis of BRSV is rarely based on the technique of virus isolation since little or no cytopathic effects are seen following inoculation to cell lines of bovine origin. The virus can be detected by immunofluorescence directly in the sampled material such as tracheal wash, nasopharyngeal swabs, tissue samples, or in inoculated cell lines (*Valarcher and Taylor, 2007; von Messling, 2016; Leme et al., 2020*). However, immunofluorescence results depend on the sampling moment and have limitations when examining field specimens due to tissue autolysis or the appearance of non-specific fluorescence (*Brodersen, 2010*). The recent study of *Kamdi et al.* (*2020*) describes the examination of samples collected from dead cattle and buffaloes under 12 months of age in India. The viruses detected by RT-PCR included BPIV-3 and BRSV, and this finding was confirmed by PCR product sequencing and direct immunofluorescence. Necrosis of the lung epithelium, thickening of the alveolar septa, and filling of the alveolar lumen with mononuclear cells and syncytial cell formations was evident in BRSV-positive lung samples. RT-PCR tests are most often used for routine diagnostic purposes, having the advantage of not being affected by the presence of neutralizing antibodies in the sampled material (*von Messling, 2016*). Accordingly, RT-PCR detects BRSV in the specimens of nasal secretions for a longer period compared to ELISA tests that are also limited by the rising titers of specific anti-BRSV antibodies (*Brodersen, 2010*). The RT-PCR method specific for the viral G protein is suitable for BRSV identification in the samples of lungs and tracheal tissue from calves with mild to severe interstitial pneumonia (*Almeida et al., 2005*). Real-time RT-PCR with

specific primers for the highly conserved viral nucleoprotein was 100 times more sensitive than the conventional RT-PCR method in the detection of BRSV in lung, trachea, and bronchoalveolar fluid samples from cattle exhibiting clinical symptoms of respiratory disease (*Boxus et al., 2005*). Still, the possible detection of viral nucleic acid from vaccinal viruses must always be considered (*Brodersen, 2010, von Messling, 2016*). *Leme et al.* (*2020*) performed the molecular characterization of G and F proteins of Brazilian BRSV field strains isolated from respiratory disease cases in 10 different cattle populations. Two strains had a high degree of similarity with analogous sequences from subgroup III, including the Bayovac vaccine strain, while the remaining Brazilian BRSV strains were different and represent a probable new virus subgroup with a mutation in the immunodominant region of the G protein. The F gene of the Brazilian viruses also bears mutations that do not exist on the F gene of other BRSV strains from the subgroups known thus far. *Krešić et al.* (*2018*) examined Croatian BRSV strains detected in the samples of nasal swabs, blood, lungs, and lymph nodes from one to six-month-old calves originating from ten cattle populations in different districts. The presence of viral RNA was not detected only in the examined blood samples. Sequencing analysis of selected BRSV strains was performed and the phylogenetic analysis showed clustering within three different genetic subgroups, namely II, VII, and VIII. These authors also revealed unique mutations within an essential immunodominant region of the viruses from subgroups II and VII. Serologic diagnosis is performed based on the detection of a 4-fold titer rise in paired serum samples, and different assays are available including virus neutralization, complement fixation, and ELISA (*Graham et al., 1997; von Messling, 2016*). Average time to BRSV seroconverison is 9 days (range 5–21 days), peaking on day 23 after infection (*Grissett et al., 2015*). *Roshtkhari et al.* (*2012*) determined the presence of BRDC-associated viruses through the examination of paired serum samples of calves with pneumonia using iELISA. During the testing period, 56.4% of blood serum samples were found to have antibodies against BHV-1, 81.5% against BRSV, 89.5% against BPIV-3. Seroconversion was found for BRSV in 16.6% and BPIV-3 in 26.1% samples, while it was not established for BHV-1.

The bovine respiratory syndrome represents a major cause of morbidity, mortality, and economic loss in farms around the world despite available therapy and immunoprophylaxis. With this in mind, appropriate and reliable laboratory diagnosis of this complex disease is very important to prevent the spread of pathogens in large animal populations. The sampling method must be properly assessed, paired with the time frame that coincides with virus shedding. Timely diagnosis is beneficial in managing and controlling BRDC, and early treatment of calves is the most important feature of a successful treatment program. It should be noted that the availability of many classical and molecular methods of laboratory

diagnostics for the identification of viruses involved in BRDC significantly contributes to the detection of infections discussed in this text.

Laboratorijska dijagnostika virusa parainfluence 3 goveda, goveđeg herpesvirusa 1 i goveđeg respiratornog sincicijalnog virusa kao uzročnika respiratornog sindroma goveda

Jakov Nišavić, Nenad Milić, Andrea Radalj, Aleksandar Stanojković, Ljubiša Veljović

Rezime

Kompleks respiratornih bolesti goveda (BRDC) nastaje kao posledica interakcije između faktora vezanih za domaćina, faktora okoline i različitih mikroorganizama. Virus kao primarni patogen najčešće dovodi do supersije imunoloških mehanizama životinje i omogućuje kolonizaciju donjih disajnih puteva bakterijama. Goveđi herpesvirus 1 (BHV-1), virus parainfluence 3 goveda (BPIV-3) i goveđi respiratorni sincicijalni virus (BRSV) su među najznačajnijim uzročnicima BRDC, pri čemu najčešće oboljevaju mlade i starije imunokompromitovane jedinke. Rezultati laboratorijske dijagnostike zavise od mesta uzorkovanja materijala i od izbora trenutka za uzimanje uzorka u odnosu na dinamiku izlučivanja virusa u spoljašnju sredinu. Uzorke za ispitivanje uglavnom predstavljaju nosni ili nazofaringealni brisevi, trahealni aspirati, tečnost dobijena bronhoalveolarnom lavažom ili tkiva. Izolacija virusa, iako predstavlja zlatni standard, oduzima dosta vremena i njena uspešnost zavisi vrste virusa, načina uzorkovanja, odnosno stanja uzorka. Većina metoda identifikacije virusa koje se koriste u današnje vreme su molekularni testovi (konvencionalni i real-time PCR ili RT-PCR) koji se brzo izvode, osetljivi su i specifični, što je od suštinske važnosti u veterinarskim dijagnostičkim laboratorijama. Sekvenciranje virusne nukleinske kiseline se upotrebljava u cilju detekcije specifičnih mutacija u virusnom genomu i molekularno-epidemiološko praćenje kretenja i širenja pojedinih virusnih sojeva. Serološka dijagnostika se zasniva na pojavi specifičnih antitela kod serološki negativnih jedinki, odnosno detekciji četvorostrukog porasta titra specifičnih antitela u parnim uzorcima krvnog seruma, a dostupni su različiti testovi, uključujući metod virus-neutralizacije, reakciju vezivanja komplementa, inhibiciju hemaglutinacije i ELISA. Pravovremeni i pouzdani rezultati laboratorijske dijagnostike su važni u cilju kontrole BRDC i predstavljaju osnovu za blagovremeno sprovođenje terapije i programa prevencije širenja infekcije.

Ključne reči: BRDC, BHV-1, BPIV-3, BRSV, laboratorijska dijagnostika

Acknowledgements

The study was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia (Contract number 451-03-68/2020-14/200143).

Author Contributions

JN and AR conceptualized the paper, which was developed further in discussion with NM, AS, and LjV. JN and AR collated articles for review, wrote and critically reviewed various drafts, NM, AS, and LjV contributed to the preparation of the final version and provided consent for submission.

Conflicts of Interest

The authors declare no conflicts of interest.

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Received 31 December 2020; accepted for publication 10 January 2021

Biotechnology in Animal Husbandry 37 (1), 17-26, 2021 ISSN 1450-9156
Publisher: Institute for Animal Husbandry, Belgrade-Zemun UDC 575.22'62
https://doi.org/10.2298/BAH2101017P

VARIABILITY OF GENETIC CORRELATIONS OF MILK YIELD AND FERTILITY TRAITS IN SIMMENTAL COWS IN DIFFERENT REGIONS OF SERBIA

Vlada Pantelić, Dragan Nikšić, Marina Lazarević, Nenad Mićić, Miloš Marinković, Nevena Maksimović, Ljiljana Samolovac

Institute for Animal Husbandry, Belgrade-Zemun, 11080 Zemun
Corresponding author: Vlada Pantelić, vladap4@gmail.com
Original scientific paper

Abstract: The Simmental breed of cattle is mostly reared in the central part of the Republic of Serbia, where it makes up about 80% of all breeds. In areas of more intensive cattle production, populations of cattle with pronounced milk yield are reared. In more extensive, as well as hilly and mountainous areas, somewhat less productive animals are raised. The main goal of this study was to examine the variability of genetic correlations of milk and fertility traits on the farms of individual agricultural producers using modern methods, depending on the breeding area, i.e. the region in which they are bred and reared. This study included 2589 controlled Simmental heifers, with lactations concluded during one year. All first calving heifers were housed and reared on agricultural family farms in the area of Central Serbia. The paper examines genetic correlations between the following traits of milk yield and fertility: duration of lactation, milk yield in standard lactation, milk fat content in standard lactation, milk fat yield in standard lactation, yield of 4% FCM in standard lactation, age at first calving, duration of service period. The results of the study of genetic correlations were obtained using mixed LSMLMW models (*Harvey 1990*). The examined genetic correlations of milk yield and fertility traits in Simmental cows showed pronounced variability depending on the breeding area where the cows are reared.

Key words: genetic correlations, Simmental breed, regions, milk yield, fertility.

Introduction

Genetic correlations are of great importance in indirect selection where changes in one trait are caused through the selection of another trait between which there is a genetic correlation. *Chaunan and Hayes (1991)* have found a moderate to

very positive 0.45 ± 0.053 genetic association between milk production and milk fat, between milk fat content and yield 0.56 ± 0.045, between milk yield and milk fat content 0.49 ± 0.050.

Campos et al. (1994), in their study of the genetic parameters of milk yield and reproductive traits of Holstein cows, have found the genetic correlation between milk yield and milk fat of 0.743; between milk yield and fat content 0.235; milk yield and duration of service period of 0.159; between milk yield and calving interval 0.170.

Estimates of genetic correlations for milk yield, milk fat and milk fat content have high interdependence according to *Miščević (1995),* which indicates that information from the first lactation can be used for selection and breeding purposes. The correlation between milk production and milk fat in the first lactation is 0.93; between milk yield and milk fat content -0.32; between milk yield and 4% FCM 0.95; between yield and milk fat content 0.49; between milk fat yield and 4% FCM 0.93 and between milk fat content and 4% FCM yield -0.42.

Marković (1999) states positive values of genetic correlations between milk traits, except for those between milk yield and milk fat content. Genetic correlation parameters range from −0.78 (milk yield and milk fat content) to 0.95 (milk yield and 4% FCM).

Costa et al. (2000), in studies related to genetic analysis of the Holstein Friesian population in the United States and Brazil, have established a value of genetic correlation coefficient between milk and milk fat production of 0.79 in Brazil and 0.62 in the United States.

Gaydarska et al. (2001) investigated phenotypic and genotypic correlations on a sample of 3,254 cows. Analyzes shows a high and positive genetic correlation between milk production and milk fat 0.935 and 0.953. The correlation between milk yield and milk fat percentage is negative -0.155. A slightly positive genetic and phenotypic association are established between the production and the percentage of milk fat of 0.171 and 0.045, respectively.

Oseni et al. (2004), in their study of genetic parameters related to the service period and the duration of pregnancy, present data on the correlation between milk production and the duration of the service period from 0.12 to 0.6.

Studying the heritability and genetic correlations of production traits of Simmental heifers in Serbia, *Pantelić et al. (2011)* find that the genetic correlation between the traits of milk yield and the duration of the service period or age at first calving is extremely weak and weak positive. Thus, the coefficients of correlation between the service period and the traits of milk yield are: duration of lactation 0.239, milk yield 0.089, percentage of milk fat 0.095, quantity of milk fat 0.105, and yield of 4% FCM 0.099. The correlation between the service period and the age at calving is 0.535. Genetic correlation between age at calving and milk traits have the following values: duration of lactation 0.245, milk yield 0.003, percentage of milk fat 0.531, quantity of milk fat 0.082 and production of 4% FCM 0.050.

In the study by *Toghiania (2012),* genetic correlations between reproductive traits and milk yield range from −0.24 to 0.593. The correlation between milk yield and calving interval is 0.593, which indicates that increased milk production is associated with a longer calving interval. The same author cites a genetic correlation between milk yield and the duration of the service period of 0.355, with higher milk yield being associated with a longer service period.

When estimating the breeding value of Holstein-Friesian cows for milk traits using the method of selection index, *Lazarević (2019)* also states the values of correlations between milk traits and fertility. Genetic correlations of milk traits and fertility are slightly higher than phenotypic correlations. There is a weak negative to almost complete positive correlation between the examined milk traits. The genetic correlation between milk yield and milk fat yield is 0.9768. The content of milk fat is in a negative genetic correlation with the yield of milk fat - 0.0055. Genetic and phenotypic correlations of milk and fertility traits are negative and very close to zero except for protein content and service period duration and protein content and calving interval which were positive: 0.1067 and 0.1010 (genetic), 0.0807 and 0, 0765 (phenotypic correlations), respectively.

Material and Methods

The Simmental breed of cattle is mostly reared in the central part of the Republic of Serbia, where it makes up about 80% of all breeds. In areas of more intensive cattle production, populations of cattle with pronounced milk yield are reared. In more extensive, as well as hilly and mountainous areas, somewhat less productive cattle breeds are reared. The parent population of the Simmental cattle breed in central Serbia consists of high quality breeding heads registered in the main cattle registry. According to the data of the Institute of Animal Husbandry, Belgrade Zemun (2019), in 2018, the number of registered heads of the Simmental breed was 163,016, which is 90% of the total number of registered cattle of all other breeds.

This study included 2589 controlled first-calving heifers of the Simmental breed, with lactations concluded during one year. All heifers were located on the farms of individual agricultural producers in Central Serbia. The paper examines the genetic correlations between the following traits of milk yield and fertility:

- duration of lactation (days) -DL
- milk yield in standard lactation (kg) -MY
- milk fat content in standard lactation (%) - MFC
- milk fat yield in standard lactation (kg) -MFY
- yield 4% MKM in standard lactation (kg) -4% FCM
- age at first calving (days) - AFC
- duration of service period (days) -DSP

The results of genetic correlation research were obtained using mixed models of LSMLMW (*Harvey 1990*):

$$Y_{ijklm} = \mu + B_i + R_j + G_k + S_l + e_{ijklm}$$

Yijklm = manifestation of the trait in the *m*th cow, daughter of the *i*th bull-sire, which produced in the *j*th region, and which calved in *k*th year in the *l*th season

μ = general average
B_i = random effect of *i*th bull-sire
R_j = fixed effect of the *j*th region
G_k = fixed effect of the *k*th year of calving
S_l = fixed effect of the *l*th calving season
e_{ijklm} = random error

The first-calving heifers included in this research were reared on family farms, but mainly in different housing and feeding conditions, depending on the breeding area. The cows were kept in barns with a tied system, on long and medium-long beds covered with straw. The diet was based on hay and whole maize plant silage, as well as ready-made mixtures of concentrates, depending on the amount of milk produced. Milk control was performed according to the AT4 method by primary breeding organizations. In the AT4 method, the measurement of the obtained quantity of milk is performed only during the morning or only during the evening milking on the control day (alternative method), where the obtained results must be mathematically corrected to the reference method.

When examining the variability of genetic correlations of production and reproduction traits by regions of Serbia, all first-calving heifers included in this research were classified into 5 breeding regions are as follows:

1. Mačva-Kolubara region (area of the municipalities of Šabac and Valjevo)
2. Braničevo-Podunavlje region (area of the municipality of Požarevac and Smederevo)
3. Šumadija region (area of the municipalities of Kragujevac and Mladenovac)
4. Zaječar region (area of the municipality of Zaječar)
5. South region (area of the municipalities of Pirot, Leskovac, Prokuplje and Niš)

Figure 1. Distribution of first calves by regions

1	2	3	4	5
342	689	737	416	405

Due to the specifics of the terrain, i.e. approximately the same configuration, nutrition, as well as the conditions and methods of keeping, the breeding areas are grouped with each other, except for the Zaječar region, which figures as independent.

Results and Discussion

In addition to examining heritability to determine optimal selection methods and procedures, it is very important to examine the phenotypic and genetic association of traits that are to be improved through selection.

Table 1. Coefficients of genetic correlations (r_p) and their errors (S_{rp}) between milk and fertility traits in standard lactation in regions 1, 2 and 3

Traits	REGION 1		REGION 2		REGION 3	
	r_p	Sr_p	r_p	Sr_p	r_p	Sr_p
DL, days						
MY, kg	-0.594	0.277	-0.469	0.206	-0.325	0.245
MFC, %	0.792	0.171	0.820	0.171	0.357	0.197
MFY, kg	-0.459	0.358	-0.324	0.231	-0.122	0.240
4%FCM, kg	-0.529	0.315	-0.387	0.221	-0.195	0.243
DSP, days	0.655	0.257	0.543	0.224	0.376	0.243
AFC, days	0.700	0.331	0.073	0.285	0.352	0.225
MY, kg						
MFC, %	-0.976	0.117	-0.292	0.279	-0.500	0.187
MFY, kg	0.989	0.023	0.982	0.011	0.956	0.021
4%FCM, kg	0.997	0.006	0.994	0.004	0.982	0.009
DSP, days	-0.937	0.199	-0.084	0.291	-0.416	0.393
AFC, days	-0.838	0.270	0.266	0.276	-0.124	0.255
MFC, %						
MFY, kg	-0.933	0.177	-0.109	0.292	0.731	0.118
4%FCM, kg	-0.958	0.142	-0.186	0.285	0.656	0.144
DSP, days	0.343	0.769	0.386	0.326	0.030	0.747
AFC, days	0.976	0.210	0.227	0.334	0.108	0.218
MFY, kg						
4%FCM, kg	0.997	0.006	0.997	0.002	0.995	0.003
DSP, days	-0.863	0.291	-0.009	0.298	0.176	0.244
AFC, days	-0.750	0.348	0.314	0.276	-0.058	0.242
4%FCM, kg						
DSP, days	-0.903	0.240	-0.041	0.295	-0.041	0.295
AFC, days	-0.797	0.304	0.295	0.275	-0.082	0.246
DSP, days						
AFC, days	0.159	0.328	0.286	0.326	0.203	0.240

Table 2. Coefficients of genetic correlations (r_p) and their errors (S_{rp}) between milk and fertility traits in standard lactation in regions 4 and 5

Traits	REGION 4		REGION 5	
	r_p	Sr_p	r_p	Sr_p
DL, days				
MY, kg	0.058	0.477	-0.417	0.267
MFC, %	-0.144	0.415	0.245	0.296
MFY, kg	-0.007	0.465	-0.374	0.272
4%FCM, kg	0.018	0.471	-0.393	0.270
DSP, days	0.239	0.281	0.244	0.236
AFC, days	0.286	0.939	-0.296	0.305
MY, kg				
MFC, %	0.194	0.323	-0.231	0.251
MFY, kg	0.924	0.050	0.981	0.010
4%FCM, kg	0.970	0.020	0.993	0.004
DSP, days	0.316	1.382	-0.396	0.952
AFC, days	0.302	0.801	-0.439	0.235
MFC, %				
MFY, kg	0.552	0.237	-0.038	0.257
4%FCM, kg	0.423	0.278	-0.118	0.255
DSP, days	0.221	0.236	0.430	1.031
AFC, days	-0.673	0.842	-0.096	0.273
MFY, kg				
4%FCM, kg	0.989	0.007	0.997	0.002
DSP, days	-0.322	1.290	-0.315	0.894
AFC, days	0.030	0.747	-0.466	0.229
4%FCM, kg				
DSP, days	-0.050	1.098	-0.350	0.918
AFC, days	0.136	0.768	-0.457	0.231
DSP, days				
AFC, days	0.194	0.323	-0.487	1.067

Coefficients of genetic correlation of milk traits, as well as individual reproductive traits - duration of service period and age at calving in different regions of Serbia, are shown in Tables 1 and 2.

Genetic correlations can be positive, when change in the additive effect of a gene in one trait causes a one-way change in the additive effect in another trait. Negative genetic correlation means opposite in the direction of changes in additive effects in two traits. The increase of the additive effect in one is accompanied by the decrease of the mentioned effect in the other trait and vice versa. The strength of the correlation of the examined traits was classified based on the interpretation of Pearson's linear correlation coefficient: ≥0.70 strong correlation, 0.30 - 0.69

mean correlation, <0.30 weak correlation, about 0.0 no linear correlation (does not exclude the existence of a nonlinear form of correlation) (www.mfub.bg.ac.rs/dotAsset/66835.pdf).

Obtained results of genetic correlations between milk yield and milk fat yield (region 1: 0.989, region 2: 0.982, region 3: 0.956, region 4: 0.924, region 5: 0.981), i.e. between milk yield and yield of 4% FCM (region 1: 0.997) , region 2: 0.994, region 3: 0.982, region 4: 0.970, region 5: 0.993) indicate the presence of a strong and complete association between these two traits. The correlation between milk yield and milk fat content had a pronounced variability ranging from -0.976 in the Mačva Kolubara region to 0.194 in the Zaječar region.

The genetic correlation between milk production and the duration of the service period by regions was in the following range: region 1: -0.937, region 2: -0.084, region 3: -0.416, region 4: 0.316, region 5: -0.396. The correlation coefficients between the service period and milk fat content were moderately strong and positive and ranged from 0.030 in the Šumadija region to 0.430 in the South region.

The coefficient of genetic correlations between age at calving with milk production had the following values: Mačva Kolubara region -0.838, Podunavlje Braničevo region 0.266, Šumadija region -0.124, Zaječar region 0.302 and South region -0.439. The mutual genetic correlation between age at calving and milk fat content showed a significant degree of variability, which ranged from -0.673 (Zaječar region) to 0.976 (Mačva Kolubara region).

Analyzing the genetic correlations of milk and fertility traits according to different regions of Serbia shown in Tables 1 and 2, we can conclude the following differences and specificities:

1. In the Mačva-Kolubara region, the correlations between the service period and age at calving, on the one hand, and milk yield, on the other, were negative and high, ranging from -0.937 to -0.838. A negative correlation between fertility and milk fat yield has also been established.

2. In the Braničevo-Podunavlje region, the correlation between the duration of the service period and the production of milk, milk fat and 4% FCM, the coefficients of genetic correlations were negative: -0.084, -0.009 and -0.041, respectively.

3. The genetic correlation between age at calving and milk yield, percentage of milk fat, quantity of milk fat, 4% FCM in the Šumadija region was negative and had the following values: -0.124, 0.108, -0.058 and -0.082, respectively.

4. In the Zaječar region, milk yield had a positive genetic correlation with reproductive traits: service period - 0.316 and age at first calving - 0.302. The correlation between milk yield and milk fat content, and 4% FCM, was positive and strong and quite high: 0.924 and 0.970, respectively.

5. The percentage of milk fat in the South region showed a negative correlation with milk yield -0.231, yield of 4% FCM -0.118, calving age -0.096 and a positive correlation with the duration of the service period 0.430 and the duration of lactation and 0.245. The genetic correlation of service period and age at calving was negative -0.487.

If we compare the obtained results with the studies of other authors, it can be concluded that the positive values of the coefficients of genetic correlations between milk yield and service period were established by *Campos et al. (1994), Petrović et al. (1998), Oseni et al. (2004), Pantelić et al. (2011)* and *Toghiania (2012)*. The positive correlation between milk yield and age at calving is stated by *Petrović et al. (1998),* who also find a positive relationship between the duration of the service period and the age at calving, on the one hand, and the yield of milk fat, on the other. A positive genetic correlation between milk yield and milk fat content, and 4% FCM, is stated by a number of authors in their studies: *Chaunan and Hayes (1991), Campos et al. (1994), Miščević (1995), Marković M. (1999), Costa et al. (2000), Gaydarska et al. (2001)* and *Lazarević (2019).*

Conclusion

The main goal of breeding and selection work is to create new generations that will surpass the previous ones in terms of their production results and show greater production effects in the production of milk and meat. For these reasons, it is necessary, in the selection work, to know the breeding value of parental pairs, as well as the degree of heredity and genetic correlation of important traits to the offspring.

Estimates of genetic correlations for milk yield, milk fat yield and milk fat content have high interdependence, which indicates that information from the first lactation can be used for selection and breeding purposes. The examined genetic correlations of milk and fertility traits in Simmental cows showed pronounced variability depending on the breeding region.

Determining the degree of correlation between two or more traits largely depends on their manifestation. Knowing the genetic and phenotypic correlations between fertility traits and milk yield can help define the breeding goal.

Acknowledgment

The results of the research presented in this paper were financed by the Ministry of Education, Science and Technological Development of the Republic of Serbia, on the basis of the Agreement on the realization and financing of scientific research work of SRO in 2021 no. 451-03-9/2021-14/200022

Varijabilnost genetskih korelacija osobina mlečnosti i plodnosti kod krava simentalske rase u različitim regionima Srbije

Vlada Pantelić, Dragan Nikšić, Marina Lazarević, Nenad Mićić, Miloš Marinković, Nevena Maksimović, Ljiljana Samolovac

Rezime

Simentalska rasa goveda najviše se gaji u centralnom delu Republike Srbije gde čini oko 80% svih rasa. U područjima intenzivnije govedarske proizvodnje gaje se populacije goveda naglašene mlečnosti. U ekstenzivnijim, kao i brdsko-planinskim područjima, gaje se nešto manje produktivna grla. Osnovni cilj ovih istraživanja bio je da se na imanjima individualnih poljoprivrednih proizvođača primenom savremenih metoda ispita varijabilnost genetskih korelacija proizvodnih i reproduktivnih osobina u zavisnosti od odgajivačkog područja odnosno regiona, u kojima se grla i odgajaju.
Ovim istraživanjem je obuhvaćeno 2.589 kontrolisanih prvotelki simentalske rase, sa laktacijama zaključenim u toku jedne godine. Sve prvotelke su se nalazile na imanjima individualnih poljoprivrednih proizvođača na području Centralne Srbije. U radu su ispitane genetske korelacije između sledećih osobina mlečnosti i plodnosti: trajanje laktacije, prinos mleka u standardnoj laktaciji, sadržaj mlečne masti u standardnoj laktaciji, prinos mlečne masti u standardnoj laktaciji, prinos 4% MKM u standardnoj laktaciji, uzrast pri prvom telenju, trajanje servis perioda. Rezultati istraživanja genetskih korelacija dobijeni su korišćenjem mešovitih modela LSMLMW (Harvey 1990). Ispitivane genetske korelacije osobina mlečnosti i plodnosti kod krava simentalske rase, pokazale su izraženu varijabilnost u zavisnosti od odgajivačkog područja gde se grla odgajaju.

Ključne reči: genetske korelacije, simentalska rasa, regioni, mlečnost, plodnost.

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Received 29 December 2020; accepted for publication 20 January 2021

Biotechnology in Animal Husbandry 37 (1), 27-43, 2021 ISSN 1450-9156
Publisher: Institute for Animal Husbandry, Belgrade-Zemun UDC 636.034'28
https://doi.org/10.2298/BAH2101027Y

WATER FOOTPRINT OF MILK PRODUCTION SYSTEMS IN SEMI-ARID PLAINS OF NORTH AFRICA

Houari Yerou[1,2], Benamar Belguerbi[2], Abdelkader Homrani[3], Kheloufi Benabdeli[2]

[1]Department of Agriculture Sciences, University of Mascara 29000, Algeria
[2]Laboratory of Geo-sciences and Sustainable Development, University of Mascara. Algeria
[3]Department of Animal Science, University of Mostaganem 27000
Corresponding author: Houari Yerou, houariyerou@gmail.com
Original scientific paper

Abstract: Water resources are becoming scarce and must be preserved. The significant use of water is linked to agriculture in general and to livestock in particular. Very little research in semi-arid regions has been devoted to assessing the contribution of ruminants to water scarcity. This contribution explores the relationships between dairy farming and the various water resources available in an ecosystem with climatic constraints. To meet future food demand while sustainably managing the available land and water resources, dairy farm systems in semi-arid regions must adapt in response to climate and socio-economic change. In this study, we focus on the south Mediterranean region to analyze the key factors influencing water productivity in dairy farming, especially in context characterized by water scarcity. In order to characterize the relationship between dairy cattle breeding and water resources, a monitoring of 40 dairy cattle stables has been carried out in a semi-arid region. The technical and economic parameters of each farm were evaluated: the use of water according to their origins to the production of fodder by source, the contribution of virtual water off the farm, the total fodder biomass, feeding system practiced on the farms and the performances achieved. Analysis of the data indicates that productivity of fodder in dry matter differ between the two systems with values of the order of 12520 to 17188 kg/ha ($p<0.05$) respectively for type extensive and intensive systems. The milk yield per cow did not exceed an average value of 3680 kg (rang 3240 to 4120 kg. The mean gross margin per kilogram of milk was low, not exceeding 0.13€. A significant effect ($p<0.05$) of the value of the water footprint between the two dairy farm systems with an average of around 2.05m^3/kg of milk (range 1.96 to 2.15 respectively for intensive and extensive farms). The contribution of rainfall is estimated at 57% and the rest is represented by the participation of irrigation and virtual water with 18% and 25% respectively. Necessary actions must be taken along the milk production process in order to improve the productivity of water for

forage production and the milk which depends in large part on annual rainfall and to a lesser extent on groundwater.

Keywords: Dairy farms, milk yield, water productivity, water footprint, semi-arid land

Introduction

Global climate change is one of the most serious challenges facing agricultural and animal husbandry in the next decades. The Intergovernmental Panel on Climate Change reported an important global warming trend from 1983 to 2012. This period was the warmest of the last 1400 years in the Northern Hemisphere. By the year 2100, an increase in global surface temperature by 3.7–4.8 °C was predicted (*IPCC, 2014*). As climate change has become a pervasive topic in global agricultural production, especially in dairy cattle breeding. These changes will result in increasingly unfavorable climatic conditions for agricultural and especially livestock production (*IPCC, 2007*, *Gauly et al., 2013*). The production husbandry systems and agricultural is of vital importance in Mediterranean region, which ensuring food security and contributes significantly to the regions' economy. According to *Kina Stientje and Žiga* (2019), in order to meet future food demand while sustainably managing available land and water resources, irrigated agriculture in semi arid regions needs to adapt as a response to climate and socio-economic change. The climatic conditions in the area are suitable for growing a wide variety of crops, irrigation is essential to maintain consistent yields (*Daccache et al.,* 2014). With around 30% of the cropland being irrigated, it is the largest consumer of freshwater in the Mediterranean region (*FAO, 2016*). Due to high population density and semi-arid climatic conditions, the Mediterranean is among the most water-scarce regions, posing serious constraints on irrigation (*Mekonnen and Hoekstra, 2016; United Nations, 2017*). water availability in the region is decreasing as a consequence of climate change, particularly due to rising temperatures and shifting precipitation patterns (*Giorgi and Lionello, 2008; Grasso and Feola, 2012; Iglesias and Garrote, 2015; Iglesias et al., 2011; IPCC, 2014*). It is estimated that the gross irrigation requirements will face an increase between 4 and 18% if irrigated agriculture does not adapt to these changing conditions (*Fader et al., 2016*). The pressure on freshwater has intensified in recent years not only due to population growth and rising food requirements but also as a cumulative impact of climate change, land cover changes, poor governance in water use, and the development of water diversions (*Sultatna et al., 2014*). At the same pace, livestock production and more specifically dairy production faces great challenges as water use in this sector is also increasing (*Khelil-Afra et al., 2012*). Livestock production, thus, impacts

heavily on the world's water supply, representing > 8% of global human water use (*Sharma, 2015; Schlink et al., 2010),* 10 % of global water flows *(Deutsch et al., 2010*), and 29% of agricultural water use (*Mekonnen and Hoekstra, 2010b*). The expansion of global dairy production has a major effect on this trend, and 19% of animal water use is already today related to dairy cattle production (*Mekonnen and Hoekstra, 2010*). However, dairying is an important source of human food and an integral part of agricultural production and the social fabric for more than two thirds of the population especially for smallholders in developing countries (*Doreau et al., 2012*). Water is used in dairy farming for producing feed crops, processing feed, watering the animals, cleaning and disinfecting the barn and equipment, and cooling the milk and the barn. Several studies have investigated water use for drinking, cleaning, and disinfection. The drinking water demand of lactating cows has been investigated by several authors, including *Cardot et al. (2008), Holter and Urban (1992), Meyer et al. (2004), Murphy et al. (1983*). All authors estimated the daily drinking water intake of cows depended on influencing factors such as the milk yield of the cows, live weight, and dry matter content of the feed, dry matter intake, day of the year, rainfall, and temperature. The water scarcity situation worldwide indicates that some areas are extremely water scarce and when it is combined with high milk production, it can be argued that water might be a threat to milk production. To address the problems of water scarcity and intensification, there is a need for research how to increase dairy production without off-setting water resource. The first step is to tackle the situation is to measure water use in dairying. In southern Mediterranean countries, water scarcity is already threatening human development (*Iglesias et al., 2007*). Algeria is exposed to climate change and water scarcity, the impact of which on forage production and technical and economic performance of dairy cattle systems are certain. A similar situation was signaled by (*Schilling et al., 2012; Srairi et al., 2015),* in North Africa where available water resources are heavily exploited, and where climate change may negatively affect the country's economy. According to *Le Gal et al. (2009)* In North Africa, dairy cattle production in semi-arid conditions is a particularly interesting system for such a study, since it implies analyzing a series of on-farm production functions, from water use for growing fodder, to its conversion into feed biomass, and efficiency diets intake by cows. Very few publications are available in the literature to clarify interaction between forage production and dairy production in semiarid ecosystems, mainly from a water use en productivity viewpoint. Such complementarities need to be addressed to assess the relative pressure of both activities on available water resources (rainfall, surface water and groundwater). Estimating water use at the various stages of animal's production and communicating those estimates will help producers and other stakeholders identify hotspots and implement strategies to improve water use efficiency. In this situation improvement in dairy cows productivity efficiency can contribute to reduce the water footprint per unit product. Though the feed

production makes up the majority of water use by ruminants, research and development efforts should focus on this area. More research and clarity are needed to examine the validity of assumptions and possible trade-offs between water use by cows and other sustainability indicators. Quantifying the water footprint of anthropogenic activities involving ruminant production is a relatively new field of research where methodologies are still developing. The term "water footprint" was coined in the early 2000s as an indicator of the volume of freshwater used to produce food (milk and meat) or an industrial product (*Hoekstra and Hung, 2002*). Although assessments using the concepts of "virtual water" and "water footprint" suggest that animal products generally have a higher water footprint than plant-based products (*Allan, 1998; Ercin et al., 2012; Mekonnen and Hoekstra, 2012*), there are large discrepancies in the values reported as well as differences in assessment methods. According to *Srairi et al. (2015)* the water productivity of dairy farms becomes a difficult task, for two reasons: the output (milk and meat) tends to vary due to different management practices, with significant difficulties in obtaining accurate on-farm data and these farms are rarely specialized in either milk or meat, suggesting that research methodologies have to deal with both products. In semi-arid contexts, sustainable water use has to be promoted, given the ongoing trend of groundwater depletion (*Wada et al., 2012*). In the case of North Africa's, increased pressure on groundwater is already threatening the sustainability of many farming systems that depend on it (*Kuper et al., 2015*). The main objective of this study therefore consists in first to estimate the water footprint of dairy farms by considering the volumes of water used their origins (rainfall, irrigation with groundwater) and virtual water, secondarily to evaluate the economic impact of water productivity in dairy farms.

Material and Methods

Water footprint in dairy cattle farms was studied in semi rid south-Mediterranean conditions. The dairy farms investigated are located in the center of Mascara town. It covers 12 municipalities, with a total area of 1401 km^2 (27.3% of the total area) and a density of population of 181 hab/km^2. It receives on average 450 mm/year with semi rid climate. It is a rich agricultural plain, known for its rain-fed farming systems (cereals, vineyard and fruit trees). The total number of farms is around of 11624 divided into 3 categories of status. The distribution of farms shows the dominance of private farms in number 8165 farms which represents 70%, with an area of 45568 ha, but the collective farms (EAC) accounted for 16%, with an area of 38157ha, in number 1890 collective farms. As well as EAI (individual farms) number of 1569 exploitation, represent 14%, with an area of 5217 ha (*Yerou et al., 2019*). A benchmark survey of dairy cattle breeders was conducted during 2019 agricultural campaign indicates that all

systems selected in this study use groundwater; with 6722 cows 60% of which is Friesian black magpie of total cows and produce 3400 ±1250 kg average milk yield per cow (*Yerou et al., 2019*).

The methodology for monitoring the sample in our study consists of a series of routine visits to 2 types of dairy farming systems, one of the intensive (type I) and the other extensive (type II). The sample followed is made up of 20 dairy farms per type to describe the water use productivity in relation with the milk yield, forages practices, dietary rations and costs of forages and milk in dairy farms. The table1 indicates the characteristics of the sample farms with diverse structural, technical parameters and strategic of use of water in dairy husbandry systems.

Each farm had an average of 14.5 ha of ARL (range = 8.6 to 16.5ha), with an average animal stocking rate of 1.84 UGB/ha of fodder crop (range = 2.1 to 3.06 UGB/ha). Feeding practice in both types of cattle farming systems is shown in Figure 1. The forage calendar of extensive type shows a use of green fodder limited only in spring, a distribution of straw in summer and which continues until the end of winter as well as a very large use of concentrated foods throughout the year. This type of calendar characterizes farms with a milk tendency based on more concentrate. But the forage calendars intensive type illustrates the use of green fodder during a large part of year round; straw is limited to only part of summer and winter. It characterizes milk-oriented farms based on fodder.

Table 1. Average structural and technical's parameters of sample farms (μ ± σ)

Parameters	Symbols	Type 1 (n=20) Intensive	Type 2 (n=20) Extensive
Surface Arable Land use	ARL	16.5 ± 1.94	8.6 ± 1.2
Forage Land (ha)	FL	6 ± 0.75	3 ± 0.73
Sorgho (ha)		1.75 ± 0.19	0.75 ± 0.21
Lucerne (ha)		1. 5 ± 0.17	0.50 ± 0.19
Barley (ha)		2.75 ± 0.15	1.75 ± 0.17
Number of Cows	NCW	12.6 ± 1.66	9.2 ± 1.45
Stocking rate/ ha fodder	UGB/ ha	2.1 ± 0.28	3.06 ± 0.23
Average milk/cow/year	APM	4120 ± 604.6	3240 ± 710.2
Milk concentrates /cow	UFLcc	5.5 ± 0.86	7.6 ± 0.78
Food cost total inputs %	FCT	62.8 ± 9.2	68.7 ± 8.7
Cost 1 liter milk (€)	PCM	0.52 ± 0.07	0.61 ± 0.09
Benefit per cow (€)	BC	881.1±132.6	618.2 ± 129.7

1 DA = 0.090 €. (DA: Dinar Algerian. €: euro).

The practice of fodder crops on the sampled farms is dictated by the availability of irrigation water (rainwater, underground), the modality of use of this water and the costs of milk production. About 41 % of the total arable land was cultivated with rain-fed (oats – *Avena sativa*; barley *Hordium vulgare*) or irrigated forage (Sorgho *Sorghum sp,* lucerne – *Medicago sativa*). In north Africa, dairy cows farms uses

also cereal straw, which is considered locally as a strategic fiber resource (*Abdelguerfi et al., 2008; Belhadia, 2016; Yerou et al., 2019*).

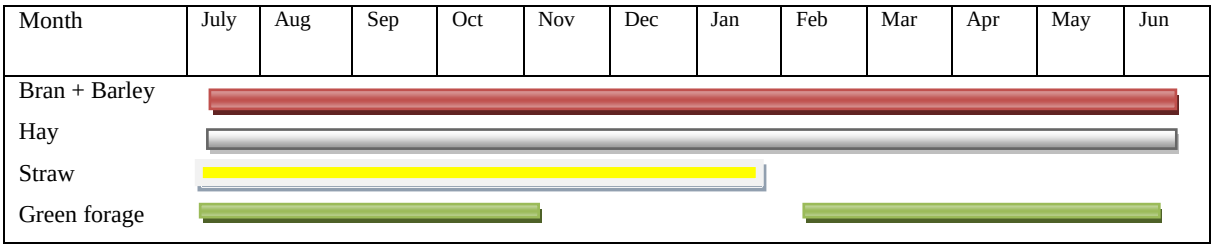

Fig 1. Forage calendar of dairy farms in study region

The parameters measured were water volumes used WU/ha of forage by cultivated species and origin of irrigation (rainfall and groundwater), estimate of forage biomass from irrigated plots and contribution of rations exogenous feeds and the milk yield obtained per systems. The estimation of the volumes of water used by breeders was based on the flow of water from the irrigation wells, the time and frequency of irrigation by irrigated forage plots and by farm. Rainfall data were obtained from the local meteorological station, which was located at a maximum distance of 9 km from farms. The exogenous rations (cereals and bran) were converted into equivalent virtual water according to international standards (*Hoekstra and Chapagain, 2007*) 1 m^3 of water per kilogram of cereals.

The method used to determine the biomass of fodder grown on the farms monitored is inspired by that cited by (*Martin et al., 2005*), which consists to weighing plant samples harvested from each plot within a 1 m^2 quadrat at each harvest. Subsequently, the nutrient value of all forages supplied by this biomass was estimated. The average dry matter (DM) and net energy content of forage crops in the context of north Africa were adopted from *Abdelguerfi et al.* (2008), INRA (2007); for all the fodder identified in the surveyed holdings in the absence of a forage analysis, we refer to other analyzes carried out in Algeria relating to these forages *Kadi et al.* (2007), *Arbouche et al.* (2009). These average nutrient values were used to calculate nutrient yields per hectare in each farm. All of the herds were on "zero grazing" and consumed distributed rations (green, dry and concentrated fodder) according to the forage management specific to each farm. The economic assessment in terms of gross margins for milk production was determined by the difference between income and expenses related to food, veterinary care and other livestock costs. Water productivity of milk production (m^3/kg) and economic water productivity of milk in (€/m^3) were also calculated.

Statistical data processing

A descriptive analysis was performed for the evaluation of averages, standard deviations, minimum and maximum of the various parameters chosen. Then a

factor analysis of variance (XL.STAT) was applied to the results according to the model: $Y_{ij} = \mu + \alpha_i + e_{ij}$; Or: Y_{ij} is the explained variable; μ: the general average, α_i : the factor effect and e_{ij}: the residual error of the model. Then, the Student test compared the factors two to two.

Results and Discussion

The objectives of the study were to characterize the use of water in the dairy farming process in semi-arid areas with irrigation possibilities and its impact on water productivity and milk production. The breeding practice within the region is characterized by poorly diversified fodder crops and a contribution of exogenous food resources to the farm which makes the analysis of these systems more complex to determine the productivity of water. According to *Kadi et al.* (2007) and *Ghozlane et al.* (2009), similar situation was reported on dairy farms in a semi-arid climate where the use of concentrates is practiced on all farms to varying degrees. The study sample was explicitly designed to represent the reality of farming in terms of structural parameters in the Mascara semiarid plain where the study was conducted (*Yerou et al., 2019*). From water volumes and their origins to forage biomass Water volumes applied to fodder crops varied widely among farms and were largely determined by irrigation practices.

The results of monitoring the two types of farming indicate that the total water use for summer fodder (Sorghum and Lucerne), was (4350; 4160) and (3150; 3050) m^3/ha respectively for in types I and II. A comparison of the irrigation practice between the two types of dairy farms indicates a variation of around 27% (7320 to 10160 m^3/ha) in favor of type I. The maximum value was recorded on Type I (intensive system), which was the only one equipped with drip irrigation. According to the precipitation recorded during our monitoring, water consumption for oats is relatively constant with an average value of 3740 and 2950 m^3 / ha respectively for the intensive and extensive systems. The productivity of fodder in dry matter differ between the two systems with values of the order of 12520 to 17188 kg/ha respectively for type II and I. This difference is due to the mode of management of the fodder crops and the volume of irrigation water applied, in the same way a relative shift of the vegetative cycles was observed within the dairy farms. Our assessment of water productivity based on the contribution of rainfall, the possibility of irrigation and the share of virtual water, indicates the existence of variability in the water used between dairy farms. The water productivity of the various cultivated forages presents a variation between farms of the order of 0.84 and 1.04 m^3/kg DM) respectively for the intensive and extensive system (Table 2). The results obtained are slightly higher than those reported by *Srairi (2009*) and *Bouazzama et al*. (2012) under irrigated conditions in Morocco (0.33 to 0.54 and 0.33 to 0.54 m^3/ kg DM).

Our results on the yield of DM from Lucerne are poor (4960 kg of DM/ha) compared to those obtained by (*Sraïri et al., 2009*) of the order of 9190 kg/ha and 6820 kg of DM/ha in a semiarid irrigated region of Morocco. This can be explained by the rainfall regime of each zone. For the water productivity of this species (0.76 m^3/kg DM) was lower than that reported by *Srairi et al. (2009), Montazar and Sadeghi (2008*).

Table 2. Water productivity of fodder in the study farms

	Type I Intensive system			Type II Extensive system		
Parameters	WU m^3/ha	DMY kg/ha	WPDM m^3/kg	WU m^3/ha	DMY kg/ha	WPDM m^3/kg
Sorghum	4350 [a]	3260 [a]	1.33[a]	3150 [b]	1460 [b]	2.15 [b]
Lucerne	4160 [a]	5458 [a]	0.76[a]	3050 [b]	4460 [b]	0.68 [b]
Barley green	1650 [a]	2600 [a]	0.63 [a]	1120 [a]	1850 [a]	0.60 [a]

a, b, means for each parameter with different letters across a row are significantly different $p<0.05$

For all the parameters characterizing the use of water, the oat crop had a relatively homogeneous use of water and a yield of DM per hectare quite similar between farms. This is explained by the technical mastery of the cultivation of this species in the northern Mediterranean production systems. With regard to the other fodder species requiring water, the technical route remains poorly controlled, which significantly affects yields by crop and exploitation within the same agro-ecological zone. In addition, profitability of using irrigation is affected by other factors relating to the mastery of the cultivation techniques of the fodder used. Similar trend results have been reported in the semi-arid North African zone with regard to the use of irrigation water (*Sraïri et al., 2009; 2016*). According to the same source, precipitation in semi-arid regions significantly affects the extent of irrigation use with an interval of the order of 82% to 87.9% of the total amount of water used to irrigate fodder crops, which implies great pressure on the groundwater of crops in regions with a semi-arid climate. In their work on dairy farming, *Meul et al. (2012)* in temperate zones indicate that the development of intensive fodder systems based on the irrigated fodder in this case maize plagues groundwater.

Relationship between distributed off-farm exogenous ration and milk performance

Monitoring the consumption of rations distributed on dairy farms indicated seasonal variability, with a peak achieved in spring (March to May), followed by regression until the end of winter. This observation is linked to the availability of

green according to the forage calendar practiced by dairy farms and the participation of exogenous distributors, which is strongly linked to the prices of the concentrated foods purchased. In addition, the quantities of DM ingested did not cover the optimal needs of the cows mainly due to the average rate of the animal load practiced 1.84 UGB/ha and the variability of the fodder yield in green realized in the semiarid conditions. The characterization of the distributed rations reveals a quantitative and qualitative imbalance of the rations which affects the efficiency of transformation of the rations into milk. The participation of the rations ingested by the dairy herds reveals that the contributions of the rations are deficient in DM. moreover, the food balance of the distributed ration is unbalanced, causing the fall in dairy performance of cows. This deficit necessitates the use of quality concentrate supplements to correct food rations. Similar observations have been reported by (*Moran, 2013; Sraïri et al., 2015*), who recommend the need to generalize the formulation of complementary feeds within dairy farms to increase milk production.

Water use productivity and profitability margin for dairy cows

In terms of feeding practice strategies, breeders always seek to reduce food production costs, by reducing the quantities of concentrates distributed during periods of green availability. This leads to a reduction in milk production per cow, although the breed exploited allows production of around 20 Kg/day under semi-arid breeding conditions. The decline in dairy performance continues during the summer period. The milk yield per cow did not exceed an average value of 3680 kg (rang 3240 to 4120 kg) (Table 3). The mean gross margin per kilogram of milk was low, not exceeding 0.13 euro.

Table 3. Virtual water use and cattle performance variability among farms

Parameters	Systems types		
	Intensive	Extensive	Average
Total WU off-farm feed uses (kg)	3780 [a]	4970 [b]	4375
Virtual water for lactation (m^3/cow)	1220 [a]	1090[b]	1155
Average milk (kg/cow per year)	4120 [a]	3240 [b]	3680
Milk profitability margin (€ / kg)	0.15 [a]	0.12 [b]	0.13
a, b, means for each parameter with different letters across a row are significantly different $p<0.05$			

In system Type I, which had the highest average annual milk yield per cow, the economic results from the herd were the highest compared at systems type II with lowest average milk yield per cow.

The estimate of the value of the water footprint at the dairy farm level indicates an average of the order of 2.05 m^3/kg of milk (with a margin varying from 1.96 to

2.15). The contribution of rainfall is estimated at 57% the rest is represented by the participation of irrigation and Virtual water with 18% and 25% respectively. This trend indirectly affects purchases of exogenous fodder resources on the farm. The results obtained in this study indicate a variation between dairy farms in terms of the percentage of dependence on rainfed crops to produce rougher fodder intended to feed their dairy herd. Economic productivity based solely on irrigation water revealed that to produce fodder for dairy barns, the use of 1 m^3 of irrigation water generates an average gross margin of around 0.17 €. Our results agree with those indicated by (*Armstrong, 2004; Sultana et al., 2014; Sraïri et al*., 2015) which report the existence of a variation between dairy farms due to the management practices of all production functions for water and livestock products.

Table 4. Water productivity characteristics in milk, in the study farms

Parameters	Systems types	
	Intensive	Extensive
Total WU per kg of milk (m^3)	2.15 [a]	1.96 [b]
Costs of total WU in milk (€ / m^3)	0.08 [a]	0.06[b]
Costs of irrigation WU in milk (€ /m^3)	0.15 [a]	0.20 [b]

a, b, means for each parameter with different letters across a row are significantly different $p<0.05$

The extensive system is the least efficient in terms of water productivity and the highest stocking rate, which considerably affects food autonomy. Moreover, within this system, poor practice in the management of fodder crops generates low water productivity in irrigated forages. Conversely, the intensive system was the most efficient in terms of milk water productivity with a lower storage rate and better performance in the productivity of water from forage crops. The comparison between the two farming systems in semi-arid region indicates that the system (type I) is characterized by greater food autonomy, allowing it to achieve good performance compared to the average of the sample studied and have the highest economic costs. The best results have been observed for the intensive system, which stands out for its good practice along the process chain, from irrigation management to farming. Intensive farming is the most specialized in milk production which is considered a strategic activity within the agricultural production system applied in the semi-arid zone. On a global scale, dairy farming systems seek to achieve food self-sufficiency to improve the gross margin per dairy barn. The scientific work of (*Val-Arreola et al., 2006; Lebacq et al., 2013; Gaudino et al., 2014; Srairi et al., 2015*) indicate that the farms with the best agro-environmental indicators and optimal management fodder resources.

This thematic contribution converges with the directions of recent research indicating the urgency of adding value to green water rather than blue water in

order to solve the problem of food security in the 21st century (*Rockström et al., 2009*). Overall, the results obtained reveal that within dairy farms in a semi-arid climate the need to assess the contribution of the various water sources integrated into the milk production process. The analysis carried out confirms the limited pressure of dairy cattle farming on groundwater, due to its dependence on rainfall and the regulation possibilities enabled by virtual water (exogenous food on the farm). In addition, pastoralists should improve forage autonomy through good control of fodder crops and off-farm food stocks, to support the sustainability of their livestock in the face of climate change affecting the semi-arid regions of North Africa. Finally, this study was carried out on farms practicing a polyculture production and dairy cattle farming system. Following the establishment of dairy cattle farming in an arid Saharan environment and due to the nonexistence of a national benchmark in terms of the use of groundwater irrigation water and its productivity in production systems in regions with climatic constraints, this contribution could help the country's authorities to better choose the irrigated perimeters and to develop non-renewable water resources in a sustainable manner in this Saharan ecosystem.

Conclusion

The study describes on the one hand, the relationship between milk production in dairy cattle stalls and the use of different water resources and on the other hand it characterizes the impact of livestock activity on the productivity in semi-arid regions. A large variation between the stables was recorded in the amount of irrigation applied according to the forage calendars practiced; this indicates that the water footprint within the farms is less effective. Other factors of variation were determined indicating a great weakness in the management of cultivated fodder and the insufficient rationing of the herds. The results reflect variability in the use of total water, whatever its origin. The activity of dairy farming in the study region depends mainly on rainfall, but supported by irrigation water whose pressure is less on groundwater compared to fodder crops. Consequently, the prospects for the resilience of dairy production systems for cows in semi-arid conditions vary from medium to good, but the improvement in water productivity remains insufficient and requires further research on the interactions between fodder crops, irrigation water and agronomic factors.

Potrošnja vode u okviru sistema za proizvodnju mleka u polusušnim ravnicama severne Afrike

Houari Yerou, Benamar Belguerbi, Abdelkader Homrani, Kheloufi Benabdeli

Rezime

Vodni resursi postaju oskudni i moraju se sačuvati. Značajna upotreba vode povezana je sa poljoprivredom uopšte, a posebno sa stočarstvom. Vrlo malo istraživanja u polusušnim regionima je bilo posvećeno proceni doprinosa preživara u nedostatku vode. Ovaj rad ispituje veze između uzgoja krava za proizvodnju mleka i različitih vodenih resursa dostupnih u ekosistemu sa klimatskim ograničenjima. Da bi zadovoljili buduću potražnju za hranom, uz održivo upravljanje raspoloživim zemljišnim i vodnim resursima, farme za proizvodnju mleka u polusušnim regionima moraju se prilagoditi na klimatske i socijalno-ekonomske promene. U ovom istraživanju fokusiramo se na region južnog Mediterana kako bismo analizirali ključne faktore koji utiču na produktivnost vode u mlekarstvu, posebno u kontekstu koji karakteriše nestašica vode. Da bi se okarakterisao odnos između uzgoja mlečnih goveda i vodenih resursa, sproveden je monitoring 40 objekata za držanje krava za proizvodnju mleka, u polusušnom regionu. Procenjeni su tehnički i ekonomski parametri svake farme: upotreba vode prema njihovom poreklu u proizvodnji stočne hrane po izvorima, količina sveže vode koja se koristi za proizvodnju proizvoda, mereno na farmi, ukupna krmna biomasa, sistem hranjenja na farmama i postignute proizvodne performanse. Analiza podataka pokazuje da se produktivnost krme u suvoj materiji razlikuje između ova dva sistema sa vrednostima reda od 12520 do 17188 kg/ha ($p<0,05$), respektivno za ekstenzivne i intenzivne sisteme. Prinos mleka po kravi nije premašio prosečnu vrednost od 3680 kg (od 3240 do 4120 kg). Srednja bruto marža po kilogramu mleka bila je niska, ne prelazeći 0,13 €. Značajan uticaj ($p<0,05$) vrednosti upotrebe/potrošnje vode između dva sistema na farmama za proizodnju mleka - prosečna vrednost od oko 2,05 m3/kg mleka (raspon od 1,96 do 2, 15 za intenzivna i ekstenzivna gazdinstva). Doprinos kiša procenjuje se na 57%, a ostatak predstavlja učešće navodnjavanja i virtuelne vode (količina sveže vode koja se koristi za proizvodnju proizvoda, mereno na mestu gde je proizvod stvarno proizveden) sa 18%, odnosno 25%. Potrebno je preduzeti neophodne korake tokom procesa proizvodnje mleka kako bi se poboljšala produktivnost vode za proizvodnju krme i mleka koja u velikoj meri zavisi od godišnjih padavina i u manjoj meri, od podzemnih voda.

Ključne reči: farme za proizvodnju mleka, prinos mleka, produktivnost vode, potrošnja vode, polu-sušno zemljište

Acknowledgments

This study was carried out by the participation of members of the research team of Geo-sciences and sustainable development laboratory (*LGDE*) of University Mustapha Stambouli of Mascara. The authors particularly thank the dairy cattle breeders of the Mascara region for their kind participation in the monitoring protocol implemented and the local agricultural authorities directing the agricultural services and the chamber of agriculture for their support.

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Received 11 June 2020; accepted for publication 10 December 2020

Biotechnology in Animal Husbandry 37 (1), 45-64, 2021 ISSN 1450-9156
Publisher: Institute for Animal Husbandry, Belgrade-Zemun UDC 636.087'65
https://doi.org/10.2298/BAH2101045S

EFFECT OF DIETARY INCLUSION OF *SILYBUM MARIANUM* OIL EXTRACTION BYPRODUCT ON GROWTH PERFORMANCE, IMMUNE RESPONSE AND CECAL MICROBIAL POPULATION OF BROILER CHICKEN

Mehdi Shahsavan, Somayyeh Salari, Mohammadreza Ghorbani

Department of Animal Science, Food Science and Animal Science Faculty, Agricultural Sciences and Natural Resources University of Khuzestan, Mollasani, Iran. P.O. Box: 6341773637.
Corresponding author: Somayyeh Salari, somayehsallary@yahoo.com, S.Salari@asnrukh.ac.ir
Original scientific paper

Abstract: The aim of this study was to evaluate the effects of *Silybum marianum* oil extraction byproduct (SMOEB) supplementation on performance and some physiological parameters of broiler chickens. Treatments were consisted of different levels of SMOEB (0%, 3%, 6%, 9% and 12% of the diet). From day 8-21, dietary inclusion of 3%, 9% and 12% of SMOEB into the diet increased ($P<0.05$) feed intake and body weight gain of broilers. From day 22-42, increasing SMOEB level increased feed intake linearly. Increasing SMOEB level increased FI linearly and quadratically from day 8-42. On day 16, a higher value was recorded for wing-web thickness in birds that were received 9% of SMOEB in the diet only at 24h following the PHA-P injection ($P<0.05$). The higher value was observed for CBH response at 12 and 24h post-injection on day 21and day 35 in broilers were fed by 9% of SMOEB supplemented into the diet for toe web thickness. It can be concluded that SMOEB could be added to the diet of broilers without any adverse effect on performance parameters and also, it can improve immune parameters of birds at levels up to 12%.

Key words: Body weight, Broiler, By- product, Immunology, Microbiology

Introduction

Due to COVID-19 crisis and drastic changes in feed chain and feed supply, search for alternative feed supplies becomes urgently apparent and needs further research (*Hafez and Attia, 2020*). Also, poultry production has become more important for developing countries following increasing human population and

demand for animal protein. According to *Longe (1986*), poultry production represents the fastest means for compensating the shortage of animal protein availability due to their fast rate of production and quick return on investment. Unfortunately, severe drought and shortage of water resources has increased the costs of conventional feedstuff production for animals in many developing countries including Iran. Therefore, low-cost by-product feeds are often used to decrease feed costs and improve farm profitability (*Rogers and Poore, 1994*). Chemical and physical properties of By-product can influence the production efficiency. For instance, the use of Agro-industrial by-products (AIBs) in animal feed can dramatically reduce the cost of feed (*Longe, 1986*). Considerable efforts have been made to improve the utilization of these AIBs in practical monogastric nutrition.

Plants have been a constant source of drugs and recently, much emphasis has been placed on finding novel therapeutic agents from medicinal plants. Today many people prefer to use medicinal plants rather than chemical drugs (*Fatehi et al., 2004*). The by-products provided through oil extraction process, which is a part of the medicinal plants production, can be used for animal nutrition.

Milk thistle (*Silybum marianum* L. Gaernt.), sometimes called wild artichoke, is a medicinal plant that has been used for thousands of years as a remedy for a variety of ailments (*Rainone, 2005*). Silybum marianum is used presently to treat alcoholic hepatitis, liver cirrhosis, liver poisoning, and viral hepatitis, and to protect the liver from side effects of medications (*Luper, 1998*). Standardized Silybum marianum extract is known as silymarin that is a combination of at least seven chemicals (*Muriel, 1992*) and it is a naturally occurring polyphenolic flavonoid (*Atanasoff et al., 2015*). Silymarin and its major constituent, silybin, have been reported to work as antioxidants scavenging free radicals and inhibiting lipid peroxidation. Some studies also suggest that they can protect against genomic injury, increase hepatocyte protein synthesis, decrease the activity of tumor promoters, stabilize mast cells, chelate iron, and slow down calcium metabolism (*Flora et al., 1998*). Flavonoids are a group of natural compounds known to have various pharmacological actions such as antioxydative, anti-inflammatory and diuretic (*Havesteen, 2002*). It has been reported that extracts of plants that are rich in flavonoids possess antimicrobial activity (*Tim and Andrew, 2005*). *Abed et al. (2015)* showed that the extracted flavonoids of Silybum marianum can act against *Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae*. In short, no study has been conducted in Iran to determine the optimal dietary inclusion level and the mode of action for these plant by-products in order to enhance the growth performance in poultry production. Therefore, the present study is aimed at investigating the efficacy of an oil extraction by-product of *Silybum marianum* on the performance, and some physiological parameters of broiler chickens.

Materials and Methods

All the procedures under taken in our study were approved by the Animal Ethics Committee at agricultural sciences and natural resources university of Khuzestan, Ahvaz, Iran.

Preparation and analysis of Silybum marianum oil extraction by-product

Silybum marianum oil extraction by-product (SMOEB) was prepared from the Barij Essence Pharmaceutical Company, Kashan, Iran. Four samples of SMOEB were prepared and were analyzed for dry matter (DM), crude protein (CP, crude fiber (CF), ether extract (EE), and ash according to AOAC (2002). Total phenolic and flavonoid content was analyzed in the study by *Senguttuvan et al., (2014).* The results are shown in Table 1.

Table 1. Chemical composition of *Silybum marianum* oil extraction byproduct (%)

DM	CP	EE	CF	Ash	Lignin	Total of Phenol*	Total of Flavonoid*	Tannin	ME[1]	NFE[2]
93	20	10	30	3.78	13.75	206.18	571.62	232.12**	1522.98	29.22

[1]Metabolizable energy value was calculated using an equation from NRC (cottonseed meal, expeller or solvent) (Janssen, 1989) (kcal/kg).
[2]Nitrogen free extract=100-(Moisture + CP + EE + CF + Ash)
*Values were analyzed according to micro/g DW
** Value was analysed according to mg/g DW

Birds, diets, and general procedures

One-day-old male chicks (Ross 308) were obtained from a local hatchery and were housed in floor pens, and were fed according to a standard broiler diet for week 1. Feed and water were provided *ad libitum* for their consumption. On day 8, totally 275 one-day-old male broiler chicks were weighed individually and were distributed randomly into 5 treatment groups, with 5 replicates (11 chicks) for each treatment group in a completely randomized design. Treatments were consisted of different levels of SMOEB (0%, 3%, 6%, 9% and 12% of diet). The feeding regimen was consisted of a starter (for day 8-21), and grower (for day 22-42) diet. The diets were formulated to meet the nutrient requirements of broilers according to *NRC (1994*). Mash feed and water were provided ad libitum throughout the experiment. The ingredients and chemical composition of the diets are shown in Tables 2 and 3. Feed was prepared weekly and was stored in airtight containers. Light intensity was kept on same level continuously for the first 3 days of post-hatching, after which a 23L:1D lighting schedule was maintained during the experiment. At day 1 of age, the temperature was set at 33°C and subsequently was reduced by 2°C/wk.

Table 2. Composition and nutrient contents of the basal diet and diets with increasing levels of *Silybum marianum* oil extraction byproduct (SMOEB) in the starter phase (d 8 to 21 posthatch)

	Levels of SMOEB (%)				
Ingredients (%)	0	3	6	9	12
Corn	58.69	56.84	54.85	51.63	48.61
Soybean meal (43% CP)	32.30	31.50	30.82	30.00	29.20
SMOEB	0.00	3.00	6.00	9.00	12.00
Wheat bran	1.76	0.87	0.00	0.00	0.00
Fish meal	3.00	3.00	3.00	3.00	3.00
Soybean oil	0.70	1.20	1.70	2.70	3.52
Dicalcium phosphate	1.26	1.30	1.33	1.36	1.35
Limestone	1.24	1.23	1.23	1.23	1.23
Common salt	0.26	0.26	0.26	0.26	0.26
Sodium bicarbonate	0.15	0.15	0.15	0.15	0.15
Vitamin and mineral premix[3]	0.50	0.50	0.50	0.50	0.50
DL-Methionine	0.14	0.15	0.16	0.17	0.18
Calculated analysis (% unless stated otherwise)					
ME (kcal/kg)	2959	2959	2959	2959	2959
Crude protein	21.21	21.17	21.18	21.15	21.15
Methionine	0.50	0.50	0.50	0.50	0.50
Lysine	1.32	1.29	1.26	1.22	1.19
Calcium	1.00	1.00	1.00	1.00	1.00
Non-phytate P	0.45	0.45	0.45	0.45	0.45
Sodium	0.20	0.20	0.20	0.20	0.20

[1] Supplied per kilogram of diet: retinyl acetate, 1.55 mg; cholecalciferol, 0.025 mg; α-tocopherol acetate, 20 mg; menadione, 1.3 mg; thiamin, 2.2 mg; riboflavin, 10 mg; calcium pantothenate, 10 mg; choline chloride, 400 mg; nicotinamide, 50 mg; pyridoxine HCl, 4 mg; biotin, 0.04 mg; folic acid, 1 mg; vitamin B_{12} (cobalamin), 1.013 mg; Fe, 60 mg; Mn, 100 mg; Zn, 60 mg; Cu, 10 mg; I, 1 mg; Co, 0.2 mg; Se, 0.15 mg.

Table 3. Composition and nutrient contents of the basal diet and diets with increasing levels of *Silybum marianum* oil extraction byproduct (SMOEB) in the grower phase (d 22 to 42 posthatch)

	Levels of SMOEB (%)				
Ingredients (%)	0	3	6	9	12
Corn	65.41	62.39	59.33	56.23	53.38
Soybean meal (43% CP)	27.71	27.00	26.27	25.62	24.71
SMOEB	0.00	3.00	6.00	9.00	12.00
Fish meal	2.00	2.00	2.00	2.00	2.00
Soybean oil	1.70	2.41	3.15	3.88	4.60
Dicalcium phosphate	0.90	0.90	0.95	0.95	0.95
Limestone	1.35	1.35	1.33	1.33	1.34
Common salt	0.20	0.20	0.20	0.20	0.20
Sodium bicarbonate	0.17	0.17	0.17	0.17	0.17
Vitamin and mineral premix[3]	0.50	0.50	0.50	0.50	0.50
DL-Methionine	0.06	0.08	0.10	0.12	0.15
Calculated analysis (% unless stated otherwise)					
ME (kcal/kg)	3100	3100	3100	3100	3100
Crude protein	18.91	18.95	18.98	19.03	19.10
Methionine	0.38	0.38	0.38	0.38	0.38
Lysine	1.13	1.10	1.07	1.05	1.02
Calcium	0.90	0.90	0.90	0.90	0.90
Non-phytate P	0.35	0.35	0.35	0.35	0.35
Sodium	0.15	0.15	0.15	0.15	0.15

[1] Supplied per kilogram of diet: retinyl acetate, 1.55 mg; cholecalciferol, 0.025 mg; α-tocopherol acetate, 20 mg; menadione, 1.3 mg; thiamin, 2.2 mg; riboflavin, 10 mg; calcium pantothenate, 10 mg; choline chloride, 400 mg; nicotinamide, 50 mg; pyridoxine HCl, 4 mg; biotin, 0.04 mg; folic acid, 1 mg; vitamin B_{12} (cobalamin), 1.013 mg; Fe, 60 mg; Mn, 100 mg; Zn, 60 mg; Cu, 10 mg; I, 1 mg; Co, 0.2 mg; Se, 0.15 mg.

Performance

Weekly body weight gain (BWG) and feed intake (FI) of each pen were recorded. Feed conversion ratio was calculated by dividing FI with BWG.

Size of different organs

At the end of the experiment, 2 birds from each replicate (which were close to the mean BW of the replicate) were selected and slaughtered to evaluate the relative weights (based on BW) of the breast, thigh, pancreas, liver, gizzard, and abdominal fat. The length of intestinal segments including duodenum, from the pylorus to the distal portion of the duodenal loop; jejunum, the segment between the point of entry of the bile ducts and Meckel's diverticulum, ileum, from Meckel's diverticulum to the ileocecal junction and cecum (left and right) were also measured separately (*Sadeghi et al., 2015*).

Apparent ileal digestibility of nutrients

The chromic oxide (Cr_2O_3) marker method reported in the study by *Vries et al., (2014)* was used to measure ileal nutrient digestibility. Marker-containing diets (supplemented with 3 g/kg of Cr_2O_3) were given for 7 consecutive days, from day 36 to day 42 of the experimental period. At the end of study, 2 birds per replicate were killed by cervical dislocation and ileal contents, from Meckel's diverticulum to the ileocecal junction, and all were collected in sealed bags. The pooled digesta samples (on pen basis) were kept frozen at -20 ˚C until further analysis was performed. Feed samples and digesta were ground (using a 0.5 mm screen) prior to chemical analysis. The samples were analyzed for dry matter, organic matter, crude protein, and ether extract according to the standard procedures of *Association of Official Analytical Chemists (2002).* Chromium oxide content in the experimental diets and digesta was measured according to the study conducted by *Saha and Gilbreath (1991).* Digestibility coefficients were calculated using the following formula (*Hafeez et al., 2016*):

Eq. 1

$$\text{Apparent Digestibility}(\%) = 100 - \left[\left(\frac{\text{Cr2O3 diet}}{\text{Cr2O3 digesta}}\right) \times \left(\frac{\text{nutrient in digesta}}{\text{nutrient in feed}}\right) \times 100\right]$$

Gut microflora

On day 28, 2 birds per replicate and 10 birds per treatment were selected and cecal digesta (1 g) from each bird were transferred aseptically into 9 mL of sterile saline solution and were diluted serially. *Lactobacilli* was enumerated on De Man-Rogosa-Sharpe (MRS) agar, *E. coli* and *coli*- form were counted on Mac Conkey (MC) agar after incubation at 37˚C for 48 h in an anaerobic chamber, and for 24 h in an aerobic chamber, respectively (*Guban et al., 2006*). All samples were plated in duplicate.

Cellular immunology

In order to assess the *in vivo* cell-mediate immune response, we prepared a solution containing 1 mg/ml of phytohemagglutinin-P (PHA-P; Sigma Chemical Co., St. Louis, MO) by adding 1 ml of sterile PBS (0.15 M at pH = 7.4) to 1 mg of PHA using the septum. At 16 days of age, 8 birds per treatment (2 birds of each replicate) were selected. Then we injected 0.1 ml of PHA-P solution either intra- or sub-dermally into the right wing web of the elbow joint (or interdigitary skin or wattle) as well as 0.1 ml of sterile PBS (0.15 M at pH = 7.4) into the left wing web as a sham control. The thickness of the wing web was measured, using digital calipers (pressure-sensitive), to the nearest 0.05 mm immediately before and at 24 hour and 48 hours after injection. The mitogen stimulation index (SI) was calculated as follows: (*Grasman 2010*). Eq 2.

The mitogen stimulation index (SI) =
[(the increased thickness of right wing web) – (the increased thickness of left wing web)]

CBH response

The CBH response to phytohemagglutinin-P (PHA-P; Sigma Chemical Co., St. Louis, MO) was used to assess *in vivo* cell-mediated immune response. At 21, and 35 days of age, 8 birds per treatment received 100 μg of PHA-P in 0.1 mL of sterile PBS (0.15 M at pH = 7.4) which was injected intradermally in interdigital skin between the second and third digits of the right foot. 0.1 mL of PBS was injected into the left foot as a sham control. The thickness was measured, using digital calipers, to the nearest 0.05 mm immediately before and at 12, 24, and 48 hours post-injection, and the response was evaluated as follows: (*Akhlaghi et al., 2013*). Eq 3.

"Cutaneous basophilic hypersensitivity response = [(thickness of right toe web postinjection − thickness of right toe web preinjection) − (thickness of left toe web postinjection − thickness of left toe web preinjection)]

Lymphoid organs

At the end of the experiment, 2 birds from each replicate were slaughtered and the spleen and bursa of fabricius were weighed and calculated as a percentage of live BW.

Statistical analysis

Data were analyzed in a completely randomized design using General Linear Model procedures of SAS (*SAS Institute, 2001*). The following model was assumed in the analysis of all studied traits: $Y_{ij} = \mu + T_i + \varepsilon_{ij}$, (Eq.4)

Where Y_{ij} is the observed value for a particular character, μ is the overall mean, T_i is the effect of the i^{th} treatment, and ε_{ij} is the random error associated with ij^{th} recording. Data were analyzed considering the pen of birds as the experiment unit regarding performance parameters, and the individual chicken was measured as the experimental unit for the rest of the parameters. The mean values were compared using Duncan's multiple range test. Statistical significance was determined at $P<0.05$. Microbiological counts were subjected to base-10 logarithm transformation before analysis.

Results

Performance

Data representing the effect of dietary inclusion of SMOEB on performance of broilers are shown in Table 4. Mortality was lower than 3% with no differences

between the groups. From day 8 to day 21, dietary inclusion of 3%, 9% and 12% of SMOEB into the diet caused the increase (P<0.05) in feed intake (FI) and body weight gain (BWG) of broilers compared to those birds that were received 6% of SMOEB. From day 22 to day 42, FI was increased (P<0.05) by the inclusion of 12% of SMOEB into the diet compared to the other groups, while BWG was not influenced. The supplementation of SMOEB at the level of 12% into diet increased (P<0.05) FI compared to the birds receiving the control diet and the diets containing 3% and 6% of SMOEB from day 8 to day 42. Orthogonal contrast comparisons (Table 4) showed that FI quadratically decreased with increasing levels of SMOEB in d 22–42 (p = 0.022) and d 8–42 (p = 0.021). The FCR improved quadratically by SMOEB supplementation (p < 0.023) from d 8 to 42. However, there were no significant differences in feed conversion ratio (FCR) between treatments, during experimental growth phases, and over the whole period of experiment.

Table 4. Effect of treatments on growth performance traits of broilers at different phases

	Levels of SMOEB (%)							Contrast	
	0	3	6	9	12	SEM	P- Value	Linear	Quadratic
Starter phase (8 to 21 d)									
Feed intake (g/b)	853.8^{ab}	880.3^{a}	799.0^{b}	878.6^{a}	900.7^{a}	21.10	0.034	0.187	0.073
Body weight gain (g/b)	430.6^{ab}	445.8^{a}	400.9^{b}	440.4^{a}	454.7^{a}	11.70	0.046	0.266	0.078
Feed conversion ratio (g:g)	1.98	1.97	1.99	1.99	1.98	0.006	0.200	1.000	0.453
Grower phase (22 to 42 d)									
Feed intake (g/b)	2773.6^{b}	2748.9^{b}	2709.6^{b}	2857.5^{b}	3104.8^{a}	76.677	0.016	0.006	0.022
Body weight gain (g/b)	1376.7	1450.1	1458.1	1471.0	1508.6	54.897	0.561	0.121	0.749
Feed conversion ratio (g:g)	2.01	1.89	1.85	1.94	2.05	0.085	0.424	0.736	0.067
Experiment overall (8 to 42 d)									
Feed intake (g/b)	3627.4^{b}	3629.26^{b}	3508.69^{b}	3736.13^{ab}	4005.57^{a}	92.063	0.018	0.009	0.021
Body weight gain (g/b)	1807.4	1895.9	1859.0	1911.4	1963.3	63.944	0.524	0.126	0.947
Feed conversion ratio (g:g)	2.01	1.92	1.88	1.95	2.04	0.049	0.196	0.583	0.023

$^{a–b}$ Means with different superscripts within the same row differ significantly (P ≤ 0.05)
SMOEB: *Silybum marianum* oil extraction byproduct

Size of different organs and length of intestine

The size of different organs is presented in Table 5. None of the dietary treatments led to remarkable changes in any of the organs (P>0.05). The length of the duodenum, jejunum, and cecum was increased in broilers fed by SMOEB at the levels of 6%, 9%, and 12% supplemented into the diet compared to control (P<0.05). The ileum length was enlarged significantly (P<0.05) at the levels of 9%

and 12% of SMOEB compared to the control group. Orthogonal contrast comparisons (Table 5) showed that length of duodenum, jejunum, ileum, and cecum linearly increased with increasing levels of SMOEB in the diet (p=0.0005, p=0.0006, p=0.001, p<0.0001, respectively).

Table 5. Effect of dietary treatments on relative organ weights (% of BW) and length of the intestine (cm) at 42 d of age in broilers

	Levels of SMOEB (%)							Contrast	
Size of different organs	0	3	6	9	12	SEM	P-value	Linear	Quadratic
Breast	21.85	21.68	23.36	21.88	22.34	0.542	0.256	0.484	0.362
Thighs	19.57	19.63	19.32	18.97	19.34	0.272	0.521	0.198	0.568
Pancreas	0.27	0.27	0.27	0.25	0.26	0.008	0.623	0.175	0.941
Liver	2.23	2.13	2.18	2.23	2.17	0.033	0.178	0.773	0.628
Gizzard	2.49	2.46	2.48	2.54	2.57	0.033	0.183	0.034	0.215
Abdominal Fat	1.60	1.50	1.501	1.82	1.92	0.147	0.184	0.052	0.197
Length of intestine									
Duodenum	27^{b}	29^{ab}	30^{a}	30^{a}	31^{a}	0.828	0.005	0.0005	0.155
Jejunum	72^{b}	79^{ab}	84^{a}	85^{a}	86^{a}	2.681	0.010	0.0006	0.110
Ileum	78^{c}	79^{bc}	89^{abc}	91^{ab}	94^{a}	4.076	0.022	0.001	0.795
Cecum	32^{b}	33^{b}	40^{a}	41^{a}	41^{a}	1.546	<0.001	<0.0001	0.145

$^{a–c}$ Means with different superscripts within the same row differ significantly ($P \leq 0.05$)
SMOEB: *Silybum marianum* oil extraction byproduct

Apparent ileal digestibility of nutrients

As shown in Table 6, apparent ileal digestibility of dry matter, organic matter, crude protein and ether extract were not influenced by the treatments. Orthogonal contrast comparisons (Table 6) showed that apparent ileal digestibility of organic matter increased quadratically by SMOEB supplementation (p=0.028).

Table 6. Effect of dietary treatments on apparent ileal digestibility of nutrients and cecal microbial population of broilers at d 42

	Levels of SMOEB (%)							Contrast	
Apparent ileal digestibility (%)	0	3	6	9	12	SEM	P-value	Linear	Quadratic
Dry Matter	77.1	77.3	77.4	77.1	76.9	0.240	0.601	0.422	0.184
Organic matter	79.5	79.9	80.0	79.8	79.5	0.159	0.231	0.724	0.028
Crude protein	72.8	72.0	72.5	72.4	72.6	0.239	0.337	0.965	0.189
Ether extract	63.1	62.6	62.9	62.8	63.0	0.144	0.216	0.960	0.068
Cecal microbial population (log CFU/g of digesta)									
Lactobacill	7.9	8.0	8.1	8.1	8.0	0.070	0.355	0.237	0.154
Coliform	7.8	7.6	7.8	7.9	7.9	0.153	0.635	0.267	0.445
E.Coli	7.8	6.9	6.8	7.1	7.1	0.357	0.348	0.331	0.153
Lactobacilli/E.coli	1.01	1.16	1.19	1.14	1.12	0.090	0.127	0.257	0.098
Total aerobes	7.7	8.0	7.9	8.1	8.1	0.130	0.176	0.078	0.459

SMOEB: *Silybum marianum* oil extraction byproduct

Intestinal bacterial colonization

The effects of SMOEB supplemented into diet on cecal microbial population of birds are shown in Table 6. Cecal population of *Lactobacillus*, *Coliform*, *E. coli*, Total aerobes and *Lactobacilli/E.coli* ratio were not influenced by the treatments (P>0.05).

Immunity

CBH response

Table 7 shows the CBH response in broiler chickens. On day 16, a higher value was recorded for wing web thickness in the birds that were received 9% of SMOEB in the diet only at 24hr following the PHA-P injection (P<0.05). Moreover, higher value of CBH response was observed in toe web thickness at 12 and 24hr post-injection on day 21 and day 35 in broilers that were fed by 9% of SMOEB supplemented into diet. The value of CBH response for wing web thickness quadratically increased (p=0.048) by increasing SMOEB levels in diet on day 16 only at 24hr following the PHA-P injection. On day 21, CBH response for toe web thickness at 12hr post-injection, linearly (p=0.016) and at 24hr (p=0.021) and 48hr (p=0.041) post-injection, quadratically, increased by supplementation of SMOEB in the diet. The CBH response quadratically affected by supplementation of SMOEB after 12hr (p=0.033) and 48hr (p=0.045) post-injection on day 35. The relative weight of bursa of fabricius was not influenced by the treatments (P>0.05; Table 7). However, higher relative weight of spleen was observed in broilers fed by 9% of SMOEB supplemented into diet compared to the other groups (P<0.05).

Table 7. Effects of dietary treatments on cutaneous basophilic hypersensitivity response to phytohemagglutinin-P (PHA-P) injection as well as the relative weight of lymphoid organs in broilers

	Levels of SMOEB[1] (%)							Contrast	
Immunity	0	3	6	9	12	SEM	P-value	Linear	Quadratic
Wing web thickness, mm (d 16)									
24h	0.20^{b}	0.23^{b}	0.23b	0.39^{a}	0.17^{b}	0.040	0.028	0.421	0.048
48h	0.19	0.21	0.21	0.32	0.16	0.052	0.343	0.840	0.225
Toe web thickness, mm (d 21)									
12h	0.27^{b}	0.31^{b}	0.37^{ab}	0.45^{a}	0.36^{ab}	0.036	0.037	0.016	0.087
24h	0.23^{c}	0.30^{bc}	0.37^{ab}	0.41^{a}	0.33^{abc}	0.031	0.032	0.014	0.021
48h	0.11	0.16	0.22	0.28	0.12	0.049	0.208	0.366	0.041
Toe web thickness, mm (d 35)									
12h	0.56^{b}	0.57^{b}	0.62^{ab}	0.71^{a}	0.55^{b}	0.033	0.032	0.343	0.033
24h	0.45^{b}	0.46^{b}	0.49^{b}	0.62^{a}	0.36^{b}	0.069	0.032	0.798	0.060
48h	0.21	0.37	0.37	0.41	0.28	0.063	0.254	0.365	0.045
Lymphoid organs [2] (d 42)									
Bursa of fabricius (%)	0.18	0.13	0.20	0.15	0.17	0.025	0.383	0.872	0.775
Spleen (%)	0.11^{b}	0.11^{b}	0.11^{b}	0.15^{a}	0.10^{b}	0.012	0.045	0.866	0.178

[a–c] Means with different superscripts within the same row differ significantly ($P \leq 0.05$).

[1] SMOEB: *Silybum marianum* oil extraction byproduct

[2] Lymphoid organs are expressed as a percentage of live body weight

Discussion

Growth performance

To our knowledge, our study is the first study investigating the effect of various levels of SMOEB on broiler performance. The literature also lacks reliable experimental data regarding this topic. We showed that from day 8 to 21, the groups that were fed by 6%, 9% and 12% of SMOEB exhibit the highest FI and BWG and those that were fed by the diet containing 3% of SMOEB as well as the control diet had the lowest level regarding these parameters. Meanwhile, from day 22 to 42 and day 8 to 42, the birds that were fed by SMOEB at the level of 12% had highest FI and there was no significant difference in the BWG between treatments. The reason for this observation is presumably the high palatability caused in part by the high concentration of SMOEB (6%, 9% and 12% of the diet). Another reason for high values of FI could be related to the lignin content of SMOEB. As shown in Table 1, SMOEB contains 13.75% lignin, and thus any increase in the levels of SMOEB, can increase the lignin content of diets. This is also supported by the findings of *Ricke et al. (1982)* which they have observed the increase in FI in broilers caused by the dietary supplementation of lignin in the diet. Also, there are some reports indicated that low concentrations of tannins increased feed intake and thus increased performance of monogastric animals (*Huang et., 2018*). The high feed intake might have enhanced body weight gain of

birds from day 8 to day 21. In our experiment, inclusion of SMOEB influenced feed consumption, but BWG and FCR were not influenced in whole period of experiment, suggesting that the primary effect of SMOEB can be on the feed intake. Similarly, Fani *Makki et al. (2013)* evaluated the ability of *Silybum marianum* seeds on performance of the broiler chickens contaminated with aflatoxin B1 (AFB_1) and they reported that *Silybum marianum* seeds increased FI and BWG, and improved FCR, however, AFB1 had a negative effect. *Gowda and Sastry (2000)* confirmed the improvement of BWG *caused by receiving Silybum marianum* seeds. They also attributed these effects to antioxidant activity that stimulated protein synthesis by the bird's enzymatic system. Perhaps the improvements in BWG of birds that were fed by high levels of SMOEB (6%, 9% and 12% of diet) from day 8 to day 21 is attributed to polyphenolic flavonoid compounds (*Atanasoff et al. 2015*) such as silymarin in SMOEB that has remained after processing by antioxidant activity. It can be pointed that flavonoid compounds like silymarin could have improved the body weight gain by influencing microbial population in digestive system (*Sťastnik et al. 2016*). Similarly, Tedesco *et al*. (2004) reported that the addition of *Silybum marianum* seeds to the broiler diets caused an improvement in body weight gain. In contrast to our findings, Suchy *et al*. (2008) reported that supplementation of *Silybum marianum* seed cakes at the levels of 0.2% and 1%, caused a decrease in BWG of broiler chickens. In the current study, both BWG and FCR were similar among birds were fed by the basal diet and the diet supplemented by different levels of SMOEB during the whole period of experiment. FCR is a measure representing how well a flock converts feed intake into weight gain. It is also the ability of the livestock to turn feed mass into body mass. Birds that have low feed conversion ratio are referred to as efficient food consumers. Presumably, due to the similar nature of SMOEB, corn, and soybean meal, broilers treated with these ingredients exhibited similar responses of BWG and FCR. As shown in Tables 2 and 3, SMOEB was replaced with corn and soybean meal in the control diet.

Size of different organs and length of intestine

Results shown in Table 5 indicate that relative weights of the breast, thigh, pancreas, liver, gizzard, and abdominal fat of broilers at 42 days were not influenced by dietary treatments. In contrast, Zahid & Durrani (2007) fed broilers by different levels of *Silybum marianum* seed, and found significantly higher breast weights at the level of 1.5 % of feed. *Fani makki et al. (2013)* investigated the interaction between different levels of *Silybum marianum* seeds (0.5% and 1%) and aflatoxin B_1 (0, 25 and 50 %) in broiler diet. They also reported that relative weight of thigh was not influenced by different levels of aflatoxin B_1 and *Silybum marianum* seeds. However, the highest weight of breast muscle was observed in birds fed by 1% of *Silybum marianum* seeds compared to the birds that were received different levels of aflatoxin B_1. *Schiavone et al. (2007)* reported that

different levels of Silymarin (0, 40 and 80 mg/kg) influenced carcass and thigh yields of broilers negatively.

The reason for the increase in length of the duodenum, jejunum, ileum, and cecum on day 42 in chickens that were received high levels of SMOEB (9% and 12%) is presumably due to the high level of crude fiber (30%) in SMOEB as shown in Table 1. This result is supported by the findings of *Sadeghi et al. (2015),* who showed the length of the jejunum was increased in broilers fed by sugar beet pulp (3%) and sugar beet pulp/rice hull compared to those received rice hull (3%). Sugar beet pulp and sugar beet pulp/rice hull enlarged the ileum significantly compared to the other dietary treatments. The amount of crude fiber in sugar beet pulp and rice hull in that study was equal to 15.1% and 44.7%, respectively. In agreement with this, *Jiménez-Moreno et al. (2013)* reported longer intestines in chicks received sugar beet pulp compared to those fed by rice hull at6 days and 12 days of age. In contrast, *Saki et al. (2011)* demonstrated that supplementing a greater ratio of soluble fiber decreased ileum length at 14 days of age. The measurements in the present study confirm that intake of high fiber diets causes a significant increase in length of the caecum. This observation has been confirmed in previous studies on birds (*Savory & Gentle 1976; Savory 1992*), and also on other animal species such as rat (*Hansen et al. 1992; Zhao et al. 1995*) and pig (*Jorgensen et al. 1996*). These changes can influence the energy metabolism as visceral organs have a high rate of energy expenditure relative to their size (*Pekas and Wray 1991*).

Apparent ileal digestibility of nutrients

Depending on the dietary fiber levels, the diet passes the digestive tract at different speeds. This speed is a crucial digestion parameter (*Mateos et al. 2012*). The coefficients of total tract apparent digestibility of nutrients, such as the dietary metabolizable energy content, can be increased significantly if fiber is included in the diet *(Gonzalez-Alvarado et al. 2010)*. Also, several studies have reported that inclusion of moderate amounts of insoluble fiber sources in the diet can increase hydrochloric acid *(Jiménez-Moreno et al., 2010*), bile acids, and enzyme production (*Hetland et al., 2003*) and result in increased nutrient digestibility and growth performance of broilers fed by low-fiber diets (*Jiménez-Moreno et al., 2009 a, b; González-Alvarado et al., 2010*). In the current study, by increasing the level of SMOEB in the diet, crude fiber level of diet was increased, as shown in Table 1, SMOEB is contained of 30% of crude fiber. The difference in apparent ileal digestibility of nutrients was almost identical between diets that were supplemented by SMOEB and the control diet.

Intestinal bacterial colonization

It is important to consider that some feed additives originating from plant products have a profound effect on gut microflora either directly or indirectly *(Cowan 1999)*. In the current study, birds fed by the diets containing different levels of SMOEB showed a similar microbial population of cecum compared to those fed by

control diet. Antimicrobial activity has been recognized as the major beneficial effect of phytogenics on animal production, although the exact antimicrobial mechanism has not been fully understood. Some *in vitro* studies demonstrated that flavonoids of *Silybum marianum* exhibited antimicrobial activity against intestinal microbes such as *Escherichia coli*, *Staphylococcus aureus*, *and Klebsiella pneumoniae* (*Abed et al., 2015*). Antimicrobial action of flavonoids is mediated by lipophilic property to perforate the bacterial membrane, which releases membrane components from the cells to the external environment (*Helander et al., 1998*). But in the current study, although, SMOEB was contained of flavonoid (as shown in Table 1) but there was not significant alterations between treatments in terms of various species of microorganisms. On the other hand, in an *in vivo* study, it seems that the findings regarding the effect of flavonoids including *Silybum marianum* on gastrointestinal microflora are not consistent with the present study, even though *Silybum marianum* has been recognized generally as an antimicrobial agent. Therefore, it is speculated that the *in vivo* antimicrobial property of phytogenic in birds can be influenced by basal diet and environmental conditions.

Immunity

The PHA-P skin response test is an *in vivo* method for measuring T lymphocyte-mediated immunity. It measures the swelling caused by inflammatory leukocyte and fluid infiltration after an intradermal injection of PHA-P. Mitogens such as PHA-P are derived from lectins, which are considered as plant or bacterial proteins that bind to specific sugar components of glycoproteins attached to the surface of cells. PHA-P specifically binds to the surface of T lymphocytes. In the skin response test, PHA-P stimulates T lymphocytes to release lymphokines, resulting in an increase in vascular permeability and the influx in a variety of leukocytes. A large increase in skin thickness indicates a strong T cell-mediated immune response (*Grasman, 2010). Ezekowitz and Hoffman (1998*) demonstrated that spleen cells are consisted of T cells, B cells, and macrophages. When antigens intrude into the body, the spleen notifies T and B cells, thus stimulating cell-mediated immunity. In the current study, the levels of 6% and 9% of SMOEB increased the influx in a variety of leukocytes to injection site of PHA-P. In addition, it had been shown that certain non-genetic factors such as dietary nutrient concentrations can alter to some extent the expression of the genes responsible for immune responsiveness *(Rama Rao et al., 2003)*. Also, cell-mediated immunity system of birds is consisted of cells with the ability of quick cleavage, which requires adequate nutrient material (*Vegad, 2002). Wang et al. (2008)* and *Brenes et al. (2008)* reported that plant extracts rich in polyphenols, as natural antioxidants are good candidates (with safety and toxicity effects), because they are obtained easily from natural sources and they can prevent lipid oxidation in food products efficiently. In the current study, the improvement in cellular immunity caused by supplementing high levels of SMOEB (especially 9% of the diet), can be partly due to polyphenolic flavonoid compounds such as silymarin in SMOEB which have remained after processing. As reported by *Basaga et al. (1997*) milk thistle

can support the immune system through its powerful antioxidant, as well as by its direct effects on immune cells. *Chand et al. (2011)* showed that Silymarin, as antioxidant, has protective action against the oxidative damages on the immune organs (bursa of fabricius, spleen and thymus). Moreover, *Mitaru et al. (1984)* reported that the lignin content of the diets appears to have some adverse effects on protein and can bind to some of amino acids such as methionine. Also, *Rama Rao et al. (2003)* reported that methionine levels lower than 0.50 % in broiler diet would generate a poorer immune cell response compared to higher concentrations. In general, the level of requirement of methionine for increased immunity is higher rather than the one for growth. Therefore, the decline in cellular immunity in birds that were fed by 12% of SMOEB can be related to the lignin content of diets. This means the lignin content of diets, only in high levels of SMOEB (12%), could suppress the cellular immunity. Similarly, as shown in Table 1, in our experiment, SMOEB is contained of 13.75 % of lignin.

Al-Khalifa et al. (2012) reported that the thymus, spleen, and bursa of fabricius are the main immune organs in poultry. During an immune response, mature lymphocytes and other immune cells interact with antigens in these tissues. Consequently, in some cases immune tissue development can indicate immune system response. *Cazaban and Gardin (2012)* showed that in young birds the bursa of fabricius plays an important role in boosting immunity. In the current study, SMOEB did not influence the growth of the bursa of fabricius. But chickens fed by diets containing 9% of SMOEB had significantly greater spleen weights compared to chickens fed by 0, 3%, 6% and 12% of SMOEB. It is not clear that why the increased spleen weight was not maintained after feeding chickens by 9% of SMOEB supplemented in diet. *Fani Makki et al. (2013)* investigated the interaction between different levels of *Silybum marianum* seeds (0.5% and 1%) and aflatoxin B_1 (0, 250 and 500 mg/kg) in broilers diet, and found out that in diets containing AFB_1, bursa of fabricius weight was decreased significantly, whereas spleen weight did not change significantly by the treatments.

According to our results, SMOEB can be used in broiler chick diets at levels up to 12%. Also, not only the use of SMOEB as by-product in the diet had not negative effect in broiler performance, but also it improved immune parameters of birds.

Acknowledgement

Special thanks to the Agricultural Sciences and Natural Resources University of Khuzestan (Khuzestan, Iran).

Uticaj uključivanja u ishranu nusproizvoda ekstrakcije ulja *Silybum Marianum* na porast, imunološki odgovor i mikrobiološku populaciju develog creva brojlerskih pilića

Mehdi Shahsavan, Somayyeh Salari, Mohammadreza Ghorbani

Rezime

Cilj ovog istraživanja je bio da se procene efekti dodavanja u ishranu nusproizvoda ekstrakcije ulja *Silybum marianum* (SMOEB) na performanse i neke fiziološke parametre pilića brojlera. Tretmani su se sastojali od različitih nivoa SMOEB (0%, 3%, 6%, 9% i 12% obroka). Od 8. do 21. dana, uključivanje 3%, 9% i 12% SMOEB u ishranu povećalo je (P<0,05) unos hrane i prirast telesne mase brojlera. Od 22. do 42. dana, povećanjem nivoa SMOEB linearno se povećavao unos hrane. Povećavanjem nivoa SMOEB povećavao se FI linearno i kvadratno od 8. do 42. dana. Veća vrednost za debljinu mrežice krila zabeležena je 16. dana kod brojlera koji su primile 9% SMOEB u ishrani samo 24 sata nakon injekcije PHA-P (P <0,05). Viša vrednost zabeležena je za CBH odgovor 12 i 24 sata nakon injekcije, 21. i 35. dana kod brojlera koji su hranjeni obrokom sa 9% SMOEB-a za debljinu mrežice prstiju. Može se zaključiti da bi SMOEB mogao da se doda u ishranu brojlera bez ikakvog negativnog uticaja na parametre performansi, a takođe može da poboljša imunološke parametre brojlera na nivoima do 12%.

Ključne reči: telesna masa, brojler, nusproizvod, imunologija, mikrobiologija

Disclosure of interest
The authors report no conflict of interest.

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Received 20 January 2021; accepted for publication 17 March 2021

Biotechnology in Animal Husbandry 37 (1), 65-73, 2021
Publisher: Institute for Animal Husbandry, Belgrade-Zemun
ISSN 1450-9156
UDC 631.563'633.32
https://doi.org/10.2298/BAH2101065D

EFFECTS OF CUTTING STAGE AND BACTERIAL INOCULANT ON QUALITY OF THE RED CLOVER SILAGE

Snežana Đorđević[1], Violeta Mandić[2], Nikola Đorđević[1]

[1]Biounik d.o.o., Research and Development Center, Šimanovci, Republic of Serbia
[2]Institute for Animal Husbandry Belgrade-Zemun, Belgrade, Republic of Serbia
Corresponding author: Violeta Mandić, violeta_randjelovic@yahoo.com
Original scientific paper

Abstract: In this paper, the influence of cut at two maturity stages (the beginning of the flowering stage and mid bloom stage and bacterial inoculant "Silko za lucerku" (contains *Lactobacillus plantarum* and *Pediococcus* spp.) on the quality of red clover silage were presented. The commercial cultivar Nada selected at the Bc Institute in Zagreb was used for investigation. The silage was examined in mini-silos (glass jars of 1.5 l volume with plastic fermentation valve) in the laboratory. The chemical composition, energy and fermentation characteristics of silages were analyzed 90 days after ensiling. The values of dry matter, acid (ADF) and neutral detergent fibre (NDF), lactic acid and pH were significantly lower, while the crude protein content, total digestible nutrients (TDN), relative feed value (RFV), ammonia nitrogen in total nitrogen (NH_3-N/TN), acetic and butyric acids were significantly higher in the first cutting stage. The inoculation with inoculant "Silko za lucerku" improved the chemical, energy and fermentation parameters of silages. Inoculant-treated silage had lower contents of ADF, NDF, NH_3-N/TN, acetic and butyric acids and pH, and higher contents of dry matter, crude protein, TDN, RFV and lactic acid than control. Accordingly, timely cutting stage and application of microbial inoculant can contribute to a lesser loss of nutritional value of the forage and promote silage quality.

Key words: bacterial inoculant, maturity stage, red clover, silage, quality

Introduction

The red clover silage is little used in ruminants' diets, although it can be a significant source of quality food like alfalfa. The alfalfa and red clover have similar crude protein, acid and neutral detergent fiber and mineral contents. However, the red clover contains polyphenol oxidases enzymes which inhibiting

plant proteases and proteolysis in the silo due to which red clover silage has more undegradable protein (25-35%) than alfalfa (15-25%) *(Hoffman and Broderick, 2001)*. It should be noted that the freshly forage of red clover contains phytooestrogen which negatively influences on development and function of reproductive organs, especially in sheep *(Hloucalová et al., 2016)*. This problem is solved by moving breeding animals from red clover crops. It has been determined that the red clover silage significantly increase performance and product quality of animal (higher milk yield, growth rate and higher amounts of isoflavonoids in milk) *(Steinshamn, 2008)*. However, the red clover as well as all forage legumes are difficult to ensile. They have high buffering capacity and low content of soluble carbohydrates *(Buxton and O'kiely, 2003)*. For that reason, it is necessary to apply chemical and bacterial additives for ensiling to ensure a stable fermentation. Essentially, bacterial inoculants improve the ensiling of legume by preventing the dry matter loss and ferment sugars to butyric acid *(Schmidt et al., 2009)* and the degradation of proteins to oligopeptides, free amino acids, ammonia and non-protein nitrogen *(Ohshima and Mcdonald, 1978)*. Lactic acid bacterial inoculant inhibits detrimental microbial activity, especially *Clostridium butyricum* in the silages *(Pys et al., 2002)*. The commercially available bacterial inoculants contain homofermentative and heterofermentative lactic acid bacteria. The homofermentative bacteria (*Lactobacillus plantarum, Pediococcus, Enterococcus and Lactococcus*) improve silage fermentation due to produced lactic acid and a faster drop in pH value *(Muck et al., 2018)*. Thus, they reduce dry matter losses and protein breakdown and the growth of undesirable microorganismsin silage. According to *Zielińska et al. (2015)*, it is recommended to use inoculants consisting of several bacterial strains because they increase content of lactic and volatile fatty acids, and aerobic stability. In general, the effect of bacterial inoculants depends on the plant species and the stage of maturity. *Mceniry et al. (2013)* found that the delaying the mowing time of red clover led to reduction of dry-matter digestibility, buffering capacity, crude protein and water-soluble carbohydrates and increasing concentrations of dry matter, neutral and acid detergent fibre. Also, *Kornfelt et al. (2013)* found the red clover silages harvested at late stage of growth has a higher dry matter, NDF, ADF and lactic acid and lower pH, acetic and butyric acids than red clover silages harvested at the early stage of growth.

The aim of this research was to examine the influence of the cutting stage and bacterial inoculant on the quality of red clover silage.

Materials and Methods

The red clover crop, genotype Nada selected at the Bc Institute in Zagreb, was taken for study. The forage was harvested at two maturity stages, at the beginning of the flowering (early; the first cut) and mid-bloom stage (late; the

second cut), in the first cut in the second year of exploitation during the growing season of 2017. After 24 h of wilting, the biomass was cut with a forage harvester to a length of about 2 cm and taken to the laboratory for testing. Two treatments were formed: "Silko za lucerku" where the green mass was treated with bacterial inoculant at a dose of 5 ml t^{-1} green mass and control where the mass was treated with distilled water 5 ml t^{-1} green mass. "Silko za lucerku" is a bacterial inoculant that contains homofermentative lactic bacteria *Lactobacillus plantarum* and *Pediococcus* spp. ($1x10^{10}$ CFU ml^{-1}). The hand-held sprayer was used to spray the inoculant and distilled water on the chopped mass. The green mass was compacted in mini-silos (glass jars of 1.5 l volume with plastic fermentation valve) and left in a dark place at room temperature around 22 ^{o}C. The silos were opened and analyzed after 90 days. Each treatment contained three replicates. The chemical composition of red clover forages at early and late growth stages are shown in the Table 1.

Table 1. The chemical composition of red clover forage before ensiling

Item	I cut	II cut
Dry matter (DM) (g kg^{-1})	280.0	480.0
Crude protein (g kg^{-1} DM)	192.7	180.7
Acid detergent fibre (ADF) (g kg^{-1} DM)	302.3	404.3
Neutral detergent fibre (NDF) (g kg^{-1} DM)	436.5	516.5

The following parameters were determined:
- dry matter content (difference in weight before and after drying at 105 ° C in a oven to a constant mass);
- crude protein content (Kjeldahl method according to *AOAC, 1990*);
- neutral - NDF and acid detergent fiber - ADF (Van Soest method according to *Van Soest et al., 1991*);
- pH value (from silage extract using a Hanna Instruments HI 83141 pH meter);
- HH_3-N/total nitrogen (distillation method using a Kjeltec 1026 analyzer);
- lactic, acetic and butyric acid (using Gas chromatograph - GC-2014, Shimadzu, Kyoto, Japan according to *Faithfull, 2002*).

Total digestible nutrients (TDN) and relative feed value (RFV) calculated according to *Horrocks and Vallentine (1999)*:

TDN (%) = (-1,291 × %ADF) + 101.35;

RFV (%) = Digestible Dry Matter (DDM) × Dry Matter Intake (DMI) × 0.775;

DDM (%) = 88.9 - (0.779 x % ADF);

DMI (%) = 120 / (% NDF).

The experiment involved two factors, each at two levels. The factorial experiment is arranged in a randomized complete block design in 3 replications. The ANOVA was used to analyze the obtained data and Tukey test for differences between mean values at the level of $p \leq 0.05$. Statistical analysis was performed with Statistical Software Package SPSS 18 (IBM Corporation).

Results and Discussion

The timing of silage cutting significantly affected the chemical, energy and fermentation parameters of red clover silages (Table 2). The dry matter content, ADF, NDF and pH significantly increased at the late cutting time for 45.1%, 22.5%, 0.5%, and 0.9% respectively, compared to early cutting time. Contrary, crude protein content, TDN, RFV, NH_3-N/TN, acetic and butyric acid significantly decreased at the late cutting time for 0.6%, 15.2%, 8.4%, 42.7%, 40.7% and 91.5%, respectively, compared to early cutting time. Similar results have also been found by *Kornfelt et al. (2013)* in red clover silage harvested at different stage of maturity. In general, the forage mass harvested at the early vegetative stage has a lower dry matter content *(King et al., 2012)* which can lead to loss of dry matter and nutrient due to the large effluent flow *(McGechan, 1990)* and increased clostridial activity in silage (*Wieringa, 1969)*. The higher protein content at the beginning of flowering result from the fact that young red clover plants have a higher share of leaves. During the vegetation period, under the influence of longer days and higher temperatures, morphological changes occur with the ageing of the plants: the leaves grow more slowly, the stem lengthens, the yield increases, and the quality decreases, especially the content of crude proteins, also, cell-wall content increases, and therefore NDF concentration *(Hatfield, 1993)*. Similar to our results, *Kuoppala et al. (2009)* found that the delaying red clover harvest resulted in reduced protein content and increased NDF content.

Table 2. Effects of cutting stage on chemical, energy and fermentation parameters of red clover silages

Item	Cutting stage (A)		F test
	I cut	II cut	
Chemical composition			
Dry matter (DM) (g kg^{-1})	238.7^{b}	434.7^{a}	**
Crude protein (g kg^{-1} DM)	175.9^{a}	174.8^{b}	*
Acid detergent fibre (ADF) (g kg^{-1} DM)	269.2^{b}	347.5^{a}	**
Neutral detergent fibre (NDF) (g kg^{-1} DM)	417.8^{b}	420.0^{a}	**
Energy parameters			
Total digestible nutrients (TDN) (%)	66.6^{a}	56.5^{b}	**
Relative feed value (RFV) (%)	150.4^{a}	137.7^{b}	**
Fermentation parameters			
pH	4.64^{b}	4.68^{a}	**
ammonia nitrogen/total nitrogen (NH_3-N/TN) (g kg^{-1} TN)	101.7^{a}	58.3^{b}	**
Lactic acid (g kg^{-1} DM)	67.9^{b}	81.8^{a}	**
Acetic acid (g kg^{-1} DM)	33.4^{a}	19.8^{b}	**
Butyric acid (g kg^{-1} DM)	4.7^{a}	0.4^{b}	**

Means followed by the same letter within a row are not significantly different by Tukey's test at the 5% level; ** - significant at 1% level of probability and * - significant at 5% level of probability.

Inoculant significantly increased contents of dry matter, crude protein, TDN, RFV and lactic acid, and significantly decreased contents of ADF, NDF, NH_3-N/TN, acetic and butyric acid and pH value compared to control (Table 3).

Table 3. Effects of inoculant on chemical, energy and fermentation parameters red clover silages

Item	Inoculant (B)		F test	
	Control	"Silko za lucerku"	B	Interaction A × B
Chemical composition				
Dry matter (DM) (g kg^{-1})	335.0[b]	338.4[a]	**	**
Crude protein (g kg^{-1} DM)	170.8[b]	179.9[a]	**	nz
Acid detergent fibre (ADF) (g kg^{-1} DM)	365.7[a]	251.1[b]	**	**
Neutral detergent fibre (NDF) (g kg^{-1} DM)	425.9[a]	412.0[b]	**	nz
Energy parameters				
Total digestible nutrients (TDN) (%)	60.6[b]	62.5[a]	**	**
Relative feed value (RFV) (%)	140.4[b]	147.7[a]	**	*
Fermentation parameters				
pH	5.02[a]	4.30[b]	**	**
Ammonia nitrogen/total nitrogen (NH_3-N/TN) (g kg^{-1} TN)	110.2[a]	49.7[b]	**	**
Lactic acid (g kg^{-1} DM)	59.2[b]	90.5[a]	**	**
Acetic acid (g kg^{-1} DM)	28.3[a]	25.0[b]	**	**
Butyric acid (g kg^{-1} DM)	4.9[a]	0.2[b]	**	**

Means followed by the same letter within a row are not significantly different by Tukey's test at the 5% level; ** - significant at 1% level of probability and * - significant at 5% level of probability.

A silage inoculant aims to supply a sufficient amount of selected strains to help optimum fermentation. The applied inoculant is a liquid inoculant contains *Lactobacillus plantarum* and *Pediococcus* spp. *Whiter et al. (1999)* pointed out that the microbial inoculants in a liquid form are more suitable for ensiling because the bacteria are added with their own moisture to help speed up fermentation. *Lactobacillus plantarum* belongs to the homofermentative lactic acid bacteria and convert sugars almost quantitatively to lactic acid. Also, *Pediococcus* spp. (belong to the family *Streptococcaceae*) ferment sugars (glucose, mannose and fructose) mainly in the lactic acid. Since these bacteria promote a rapid fermentation, the pH drops sharply in the inoculated silage and is prevented enterobacteria from breaking down the protein, as indicated by the lower ammonia content. *Kung and Muck (1997)* reported that in more than 60% of studies, the silages treated with homofermentative lactic acid bacteria had a lower pH, NH_3-N/TN, acetic and butyric acids and higher lactic acid content. Contrary, lactic acid fermentation in the control silage is slower because it is regulated only by epiphytic bacteria whose number in the forage before ensiling is small. According to *Schmidt et al. (2009)* and *Zhang et al. (2009)*, their number is less than 1% microbiome. The rate of 1 x 10^5 CFU per gram of fresh forage will provides enough microorganisms for good

fermentation *(Cai et al., 1998)*. "Silko za lucerku" has a higher level than this, which means it has sufficient bacteria to positively influence silage fermentation. It is considered that if a silage inoculant has a lower level than this, or does not even specify a CFU count, then there may be insufficient bacteria to influence silage fermentation.

Only the crude protein content and NDF content were not significantly affected by the interaction of cutting time and application of inoculant. The lower values of ADF, NDF, pH, NH_3-N/TN, acetic acid and butyric acid and the higher values of dry matter, crude protein, TDN, RFV and acetic acid were recorded at both cutting in treatment "Silko za lucerku". These results indicated that the red clover forages independently of harvest time could be well fermented as good quality silage using lactic acid bacteria inoculant, such as "Silko za lucerku".

Conclusions

The red clover silage harvested at the mid-bloom stage has a higher dry matter, ADF, NDF, lactic acid and pH and lower crude protein, TDN, RFV, NH_3-N/TN, acetic and butyric acids than red clover silage harvested at early flowering stage. The aplied inoculant containing homofermentative lactic acid bacteria positively affected the silage's chemical, energy and fermentacion parameters during the fermentation process. The inoculant enhanced the dry matter, crude protein, TDN, RFV and lactic acid content and decreased the NDF, ADF, NH_3-N/TN, acetic and butyric acids and pH, which resulted in a better silage quality.

Acknowledgements

The results of the research presented in this paper were financed by the Development Agency of Serbia (No 1-05-401-1068/2017) and Ministry of Education, Science and Technological Development of the Republic of Serbia, on the basis of the Agreement on the realization and financing of scientific research work of SRO in 2021 no. 451-03-9/2021-14/200022.

Uticaj faze košenja i bakterijskog inokulanta na kvalitet silaže crvene deteline

Snežana Đorđević, Violeta Mandić, Nikola Đorđević

Rezime

U radu je ispitivan uticaj dve faze košenja crvene deteline u proleće u drugoj godini eksploatacije (početak cvetanja (rana) i puno cvetanje (kasna)) i bakterijskog inokulanta "Silko za lucerku" (sadrži *Lactobacillus plantarum* i *Pediococcus* spp.) na kvalitet silaže. Za istraživanje je korišćena komercijalna sorta Nada selekcionisana u BC Institutu u Zagrebu. Silaža je analizirana u mini silosima (staklene tegle zapremine 1,5 l) u laboratoriji. Hemijski sastav, energetski i fermentacioni parametri silaže analizirani su 90 dana nakon siliranja. Vrednosti suve materije, kiselih (ADF) i neutralnih deterdžentskih vlakna (NDF), mlečne kiseline i pH bile su značajno niže, dok su sadržaj sirovih proteina, ukupna svarljiva hranljiva materija (USHV), relativna hranljiva vrednost (RHV), sadržaj amonijačnog azota u ukupnom azotu (NH_3-N/TN), sirćetne i buterne kiseline bili značajno veći u prvoj fazi košenja. Inokulacija sa inokulantom "Silko za lucerku" je poboljšala hemijske, energetske i fermentacione parametre silaža. Silaža tretirana inokulantom imala je niži sadržaj ADF, NDF, NH_3-N/TN, sirćetne i buterne kiseline i pH, i više suve materije, sirovih proteina, TDN, RFV i mlečne kiseline od kontrolne. Prema tome, pravovremena faza košenja useva i primena mikrobiološkog inokulanta može doprineti manjem gubitku hranljive vrednosti krme i promovisati kvalitet silaže.

Ključne reči: bakterijski inokulant, faza razvića, crvena detelina, silaža, kvalitet

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Received 5 February 2021; accepted for publication 22 March 2021

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Milan M. Petrović[1], Stevica Aleksić[1], Milan P. Petrović[1], Milica Petrović[2], Vlada Pantelić[1], Željko Novaković[1], Dragana Ružić-Muslić[1]

[1]Institute for Animal Husbandry, Belgrade – Zemun, 11080 Zemun, Serbia
[2]University of Belgrade, Faculty of Agriculture, Nemanjina 6, 11080 Zemun, Serbia
Corresponding author: Milan M.Petrović, **e-mail address**
Review paper

Example 2

EFFECTS OF REARING SYSTEM AND BODY WEIGHT OF REDBRO BROILERS ON THE FREQUENCY AND SEVERITY OF FOOTPAD DERMATITIS

Zdenka Škrbić, Zlatica Pavlovski, Miloš Lukić, Veselin Petričević

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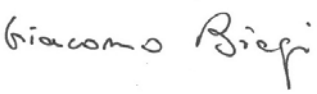

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