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Original article

EFFECTS OF THE HERBAL PREPARATION AYUFERTIN, USED FOR ANESTRUS OVERCOME, ON FATTY ACIDS COMPOSITION OF MILK IN BULGARIAN MURRAH BUFFALOES

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Summary

Ilieva, Y., D. Mihaylova, A. Ilyazova, P. Penchev, D. Abadjieva & E. Kistanova, 2020. Effects of the herbal preparation AyuFertin, used for anestrus overcome, on fatty acids composition of milk in Bulgarian Murrah buffaloes. *Bulg. J. Vet. Med.* (online first).

The herbal protocols, used instead of hormones in buffalo breeding for reproduction optimisation, do not provide information of their effect on the milk quality of treated animals. This research analysed whether the herbal preparation AyuFertin (Indian Herbs Specialities Pvt. Ltd, Saharanpur), applied in postpartum period for recovering the ovary cycling, had a side effect on the milk composition. The experiment was conducted with buffaloes of the Bulgarian Murrah breed at average age of 50 months during May-June 2019, divided into control (n=6) and experimental (n=7) groups. The experimental buffaloes received AyuFertin orally for three consecutive days at a dose of 3 g/100 kg live weight since the 21st postpartum day. The animals without signs of estrus were treated again after 10 days. The milk samples were collected at test-days, before and after treatments. The fatty acid composition of the milk and Ayufertin was evaluated by gas chromatography-mass spectrometry analysis. Quantification of the identified fatty acids in milk was made by the area normalisation method. In general, the fatty acids composition did not differ after the treatment with AyuFertin. Deviation in the amounts of some fatty acids was established. The content of medium-chain fatty acids such as lauric and carpic was decreased, while the amount of long-chain acids such as 11-hexadecanoic and stearic was increased in the experimental group. The main active components of AyuFertin – trienoic acids were not transferred into the milk of treated animals. In conclusion, due to the ability of AyuFertin to affect the fatty acids amount in the milk of treated buffaloes, farmers should strictly follow the manufacturer's recommendation for the dose and duration of AyuFertin treatment to avoid negative effects on milk quality.

Key words: AyuFertin, buffaloes, herbs, milk components, trienoic fatty acids

INTRODUCTION

Monitoring of dairy products for food safety hazards in the dairy supply chain is very important for consumers' health. Most hazardous compounds enter the dairy chain at primary production, either through the ingestion of contaminated feed, via the uptake of compounds as a result of grazing or via the administration of veterinary drugs (van Asselt *et al.,* 2017). Different plant specific compounds may be also transferred into the milk. Some of them are useful, whereas others pose a human health risk due to the genotoxic, carcinogenic or pro-inflammatory properties of the compounds (Hoogenboom *et al.,* 2011).

Buffalo milk is an important and valuable product, rich in bioactive compounds (vitamins, minerals, monounsaturated fatty acids) that may promote bone and heart health and protect the body from oxidative stress (Khan *et al*., 2019). Buffalo milk is characterised by a higher content of lactose, protein, as well as the presence of biliverdin, bioactive pentasaccharide and gangliosides, which are not present in cow's milk (Abd El-Salam & El-Shibiny, 2011).

With regard to animal welfare and organic production, many herbal protocols have been used instead of hormones or in combination with them to overcome the postpartum anestrus in buffaloes (Gupta *et al.,* 2011). AyuFertin, a non-hormonal and completely herbal preparation, is a product used for this purpose. It is recommended for the treatment of the postpartum and postpubertal anestrus conditions in livestock. The preparation contains *Piper longum, Piper nigrum, Zingiber officinale, Citrullus colocynthis*, which are well known in nontraditional medicine (Sahrajabian *et al.,* 2019).

Piperine, a major alkaloid of *Piper* species, which possesses cytoprotective and antioxidant activity, antiproliferative, anti-inflammatory, and neuropharmacological activities, may protect the liver and increase the levels of noradrenaline and serotonin in the brain (Moghadamnia *et al.,* 2010). *Z. officinale* is a strong antioxidant and may either mitigate or prevent generation of free radicals. The main pharmacological ingredients of ginger such as zingerone, gingerdiol, zingibrene, gingerols have immunomodulatory, anti-inflammatory, antiapoptotic, antihyperglycemic, antilipidemic properties (Ali *et al.,* 2008; Al-dain Qussay *et al.,* 2015). In addition, phenolic compounds, saponins, flavonoids, alkaloids, tannins, glycosides, triterpenoids, steroids and specific cucurbitacins are present in *C. colocynthis*. As far as its medicinal significance is concerned, only antioxidant activity, antihyperlipidaemic, antimicrobial and analgesic effects are reported till now (Gurudeeban *et al.,* 2010; Dhakad *et al.,* 2017).

The mentioned herbs contain various fatty acids, including trienoic fatty acids, which are considered to be the main active components of AyuFertin affecting the reproductive system as precursors of prostaglandins biosynthesis (Limem *et al.,* 2016; Oforma *et al.,* 2019). In addition to their regulatory role in the reproductive system, prostaglandins act as mediators and regulators of inflammation, affecting both pro- and anti-inflammatory pathways (Tilley *et al.,* 2001). However, the accumulation of trienoic fatty acids in milk may have a negative effect on consumers as it provides the body with more substrates for prostaglandins synthesis and disturbs the balance between pro- and anti-inflammatory processes (Bordoni *et al.,* 2017).

The data on the effect of *P. longum, P. nigrum, Z. officinale* and *C. colocynthis* as well as whole preparation Ayufertin on the quality of buffalo's milk in the scientific literature are limited and insufficient. The present investigation aimed to analyse whether AyuFertin, used for optimisation of reproduction in buffaloes postpartum had side effects on the fatty acids composition and their amounts, in particular those of trienoic acids, in the milk of treated animals.

MATERIALS AND METHODS

Animals

The experiment with AyuFertin treatment was organised at the farm of the Agricultural Institute, Shoumen, for Bulgarian Murrah postpartum buffaloes at average age of 50 months during May–June 2019. The experimental work with animals in the farm was approved by the National Ethics commission for animals in accordance with the Bulgarian Veterinary Law (25/01/2011) regarding the life conditions and welfare of livestock animals used for experimental purpose, adapted to the European Union Regulation 86/609 (AF 9747A-0002 / N1430 from 05.04.2018).

The animals were divided into two groups, control (n=6) and experimental (n=7), distributed by the method of analogues. Both groups of animals received standard daily diet for dairy animals: 25 kg of green forage (vetch, oat, sorgum), 4.5 kg of concentrate feed and wheat straw *ad libitum*. The experimental animals were administered orally with the feed supplement AyuFertin (Indian Herbs Specialities Pvt. Ltd, Saharanpur, India) for three consecutive days at a dose of 3 g/100 kg live weight in accordance with producer's instructions. The detection of estrus was done by a fertile bull. For animals that did not manifest estrus, the same dose was repeated after 10 days.

Milk samples collection

The milk samples from both groups of animals were collected at milk test-days every month in addition to milk samples obtained before and after the treatment from the experimental group. The milking of buffaloes was done twice daily. Individual milk production was recorded with electronic weighing-machine and milk yield measured in kg per day. The milk from the morning and evening milking obtained from each buffalo was collected and mixed, obtaining a representative individual milk sample. The test-day samples from both groups were sent in the laboratory for the routine detection of protein (%) and fat (%) by infrared analysis with MilcoScan-FT1. The samples from the experimental group were freeze-dried and kept until analysis processing.

Analyses of total fatty acids content and fatty acids composition

The fatty acid composition analysis was performed on the milk samples and the Ayufertin supplement.

The extraction of the fatty acids was carried out by means of diethyl ether in a Soxhlet apparatus following AOAC Method 920.39 (2012). The fatty acids methyl esters (FAMEs) were prepared in accordance with ISO 12966-2:2017 (2017).

GC-MS analyses of the whole fatty acids content was performed using a Thermo Scientific GC-MS system (Thermo Scientific, Waltham, MA, USA) comprising an AI/AS 1310 auto-sampler and a Gas Chromatograph (Trace 1300) interfaced to a ISQ mass spectrometer, equipped with TR-5MS fussed silica capillary column

(30 m × 0.25 mm, ID 0.25 μm). For GC-MS detection, electron impact mode was used. Mass spectra were taken at 70 eV, a scan interval of 0.2 s and m/z range 46 to 650 Da. Helium gas was used as a carrier gas at a constant flow rate of 1 mL/min, and an injection volume of 1 μL was employed (a split ratio of 1:25). The MS transfer line temperature was set to 260 °C and the ion-source temperature was 220 °C. The oven temperature was programmed from 110 °C, held for 3 min, with an increase of 10 °C/min to 220 °C, held for 6 min, then 15 °C/min to 310 °C and held for 5 min. The injector temperature was maintained at 240 °C.

The identification of the individual FAMEs was made by comparison of their mass spectra with NIST standard reference database. The spectra of the unknown components were compared with the spectrum of known components stored in the NIST library.

Quantification of the identified fatty acids in milk samples was made by the area normalisation method (represented as % of the area of total fatty acids in the sample).

Statistical analysis

The statistical analysis of the results was done by software product Stat.Soft, v.10 (StatSoft Inc., Tulsa, USA). Due to small number of used animals, a non-parametric statistical method was applied. The significance of the difference between samples' means in the same group were determined by the non-parametric Wilcoxon signed rank test and between groups by the Mann Whitney test. The differences were considered significant at $P<0.05$. Data are presented as a mean ± standard error of the mean values (SEM).

RESULTS

The supplementation of the animals with Ayufertin was performed in May 2019. Two animals exhibited estrus after the first treatment and other five – after the second. In June, a tendency towards increased milk yield and fat content and decreased protein content in milk samples of both groups was observed (Table 1). However, significant changes in milk fat and protein content compared to May were established only in the the experimental group.

Comparison between the fatty acid composition in the Ayufertin supplement and in the milk samples of the treated buffaloes showed that in both groups of samples there were similar two long-chain acids, palmitic and stearic (Table 2). However, the trienoic acids found in the Ayufertin were not established in the milk of the treated buffaloes. In addition, the composition of fatty acids in milk samples did not differ before and after treatment

Table 1. Milk productivity parameters (mean±SEM) in control buffaloes and buffaloes supplemented with Ayufertin (experimental) at test-days in May and June.

Groups		Milk yield, kg/day	Milk fat, %	Milk protein, %
Control (n=6)	May	7.52±0.65	7.77±0.31	5.01±0.05
	June	7.70±0.41	8.23±0.35	4.86±0.07
Experimental (n=7)	May	8.87±0.85	7.57±0.13	5.12±0.04
	June	9.16±0.61	8.26±0.27*	4.90±0.03*

* $P<0.05$ compared to the mean values of the group in May.

Table 2. Comparitive analysis of fatty acids detected in Ayufertin and buffaloes' milk ("+" present and "–" absent)

Fatty acids	AyuFertin	Milk of buffaloes treated with Ayufertin (n=7)
Capric acid (10:0)	–	+
Lauric acid (12:0)	–	+
Myristic acid (14:0)	–	+
Pentadecanoic acid (15:0)	–	+
11-hexadecanoic acid (16:1)	–	+
Elaidic acid (18:1 n-9 cis)	–	+
Palmitic acid (16:0)	+	+
Stearic acid (18:0)	+	+
Palmitin monoglyceride	+	–
Linoleic acid (18:2)	+	–
Oleic acid (18:1 n-9 *trans*)	+	–
6,9,12-Octadecatrienoic (gamma-linolenic) acid (18:3 n-6,9,12 *all cis*)	+	–
9,12,15-Octadecatrienoic (alfa-linolenic) acid (18:3 n-3,6,9 *all cis*)	+	–

Table 3. Total and individual fatty acids content in milk samples from the experimental buffaloes in pre- and post- treatment periods. Data are presented as mean±SEM (n=7).

Fatty acids	Pre-treatment period	Post-treatment period (after one dose Ayufertin)	Post-treatment period (after two doses Ayufertin)
Capric acid (10:0)	1.95±0.56	0.82±0.05**	1.43±0.18
Lauric acid (12:0)	3.40±1.02	1.70±0.14**	1.98±0.15*
Myristic acid (14:0)	14.06±3.52	11.30±1.30	12.37±1.35
Pentadecanoic acid (15:0)	1.20±0.91	0.56±0.08	0.66±0.05
11-hexadecanoic acid (16:1)	0.68±0.09	0.77±0.04	1.00±0.06**
Palmitic acid (16:0)	41.54±3.18	38.39±2.20	39.28±2.11
Elaidic acid (18:1)	22.87±4.37	27.24±2.60	23.24±2.73
Stearic acid (18:0)	14.45±0.77	17.61±0.80**	20.05±1.01**
Total fatty acids content	34.61±4.11	33.70±0.47	31.82±0.60

* $P<0.05$, $^{**}P<0.01$ compared to the pre-treatment period.

(Table 3). The total fatty acids content was found to be relatively stable and varied between 34.61±4.11 and 31.82±0.60% before and after the second treatment period, respectively. However, after the addition of AyuFertin, significant changes in the amount of some fatty acids were found. After treatment the amount of medium chain acids such as lauric and carpic acids in milk decreased, while the amount of long-chain acids such as 11-hexadecanoic and stearic acids increased regardless of the dose of AyuFertin taken (Table 3).

DISCUSSION

Milk is one of the oldest foods known to people, a complex mixture of fat, proteins, carbohydrates, minerals, vitamins and other miscellaneous constituents dispersed in water (Ozrenk & Inci, 2008). The quality of milk is reliant on milk composition that varies with stage of lactation, breed, environment, dietary composition and season (Uallah *et al*., 2005; Kittivachra *et al.,* 2007). The results of the present investigation showed that the milk productivity parameters recorded at the test-days in May and June followed similar trends of change in both control and experimental groups: a slight increase in milk yield and fat and decrease in milk protein. The effect of season in a broader sense results from the interaction of various factors throughout the year (Malacarne *et al.,* 2005). The variation of buffalo milk composition between May and June in the current research and that of the fat content in particular, was in agreement with data of other authors who reported the highest percentages of fat and solids in buffalo milk during the summer (Bhonsie *et al.,* 2003). The fat content established in this study within 7–8% was similar to that reported by Varricchio *et al*. (2007) in Mediterranean Italian breed and Thomas (2008) in Murrah breed, while the protein content (4.86–5.12%) was higher than the results reported by Thomas (2008) – 4.31%.

Many authors pay attention to the importance of feed additives in buffaloes' diet, which lead to improvement of milk yield, milk protein, milk fat, somatic cell counts, they may also improve health status and decrease the incidence postpartum diseases and ensure a better reproductive performance (Kumari & Akbar, 2006; Tufarelli *et al.,* 2008; Nawaz *et al.,* 2009; Chandra *et al.,* 2017), similarly to the established effect of the tested supplement AyuFertin.

Kolte *et al.* (2008) reported that herbal preparations altered effectively milk constituents and increased the milk production in cows with sub-clinical mastitis. Qureshi (1999) not only observed an increase in milk yield but also an increase in the fat percentage of milk in dairy cows fed the herbal combination Lectovet. Another non-hormonal herbal preparation – Galactin Vet, significantly improved milk production in dairy animals (Holstein and Jersey crosses) and increased fat percentage, affecting the economics of dairy products (Ramesh *et al.,* 2000; Baig & Bhagwat, 2009). High milk yield was obtained from animals (cattle and buffaloes) fed Ruchamax, a supplement containing 28 different herbs, which optimised the digestive functions and led to increase in milk production (Pradhan *et al.,* 1994; Singh *et al.,* 1996). Chandra *et al.* (2017) concluded that a mix of six herbs, one of which *Z. officinale*, had a beneficial effect on buffalo milk production and udder health during the transition period. Our results are in line with the findings of Kholif *et al.* (2012) and Al-Musodi & Jaafar (2019) who reported that feeding goats with *Z. officinale* significantly improved milk yield in supplemented groups as compared to control groups. In addition, some studies indicated that ginger root stimulated the appetite of animals, improving the nutrients' palatability (Tilgner, 1999) and had favourable effects on digestion and absorption of lipids (Patel & Sirnivasan, 2000).

In this study, the tendency to increased milk yield in the experimental group in June could be due to the galactopoietic activity of some of the herbs in AyuFertin. *C. colocynthis* (L.), which was reported to be useful for controlling mastitis, has been used as a galactagogue (Takhar, 2004; Raihan *et al.,* 2010). Furthermore, *C. colocynthis* mixed with *Euphorbia convolvuloides* or with other plants, applied to the breasts or given by infusion, can increase milk production and milk quality (Schmelzer & Gurib-Fakim, 2008).

According to Sadou *et al.* (2007) two of the principal saturated fatty acids of *C. colocynthis* are palmitic (8.1–17.3%) and stearic acids (6.1–10.5%), which can explain the higher content of these two fatty acids in the milk of treated animals as compared to other studies on the milk of Bulgarian Murrah buffaloes (Penchev *et al.,* 2016). The higher level of stearic acid might be due to its diminished desaturation to oleic acid in the mammary gland (Loften *et al.,* 2014) or to the reduced ruminal biohydrogenation of oleic and linoleic acids to C18:0 (Palmquist *et al.,* 2005; Lee *et al.,* 2011). In addition, Boerman *et al.* (2017) pointed out that supplementation with C18:0 resulted in a moderate increase of C18:0 in milk, and this increase was enough to positively affect the milk fat content in the experimental group in June. Moreover, long-chain fatty acids (C18) such as stearic acid, are almost exclusively derived from blood plasma fatty acids of dietary origin (Linn, 1988), and a significant increase could be attributed to the addition of AyuFertin that contains stearic acid in accordance with our findings. The results illustrated that the saturated fatty acids were predominant in the buffalo milk fat in agreement with data of Varricchio *et al.* (2007) in both pre-and post-treatment milk samples.

The content of medium-chain fatty acids such as lauric and capric in our control samples was similar to the data reported for buffalo milk by other researchers (Bergamo *et al.,* 2003; Abd El-Salam & El-Shibiny, 2011). However, a reduction was established in buffalo milk after consumption of AyuFertin. According to our results, the first dose of the preparation had the most significant effect, followed by a reduced strength of the effect after the second addition. The mechanism of this action is still unclear. However, lauric and capric fatty acids have health-promoting effects on humans through metabolic benefits associated with dissolving cholesterol deposits (Forouhi *et al.,* 2014) and inhibiting the growth of bacteria and viruses (Thormar *et al.,* 1994; Sun *et al.,* 2003). Therefore, the lower content of medium-chain fatty acids may reduce the health benefits of milk for consumers.

The trienoic acids in the composition of AyuFertin supplement were not established in the milk samples, which could be attributed to their metabolism. These results are in agreement with data of some authors reporting that trienoic acids were found only in traces in milk (Gunston, 2003; Jahreis & Dawczynski, 2020), and are particularly difficult to be detected in early lactation (Smith & Jack, 1953). Animals, like humans, cannot produce trienoic acid alone, such as linolenic acid (9,12,15-octadecatrienoic acid), therefore it is an essential part of the diet that should be obtained from plant sources in appropriate quantity. After ingestion, this acid is metabolised to the essential C20:5 eicosapentaenoic acid and C22:6 docosahexaenoic acid, and C20 acid is a precursor to prostaglandin biosynthesis (Gunstone, 2003). Therefore, the complete absence of trienoic acids in milk of treated animals is a positive feature of milk quality and may indicate that these acids have been successfully metabolised and used to affect the reproductive system. In other cases, the consumption of milk high in trienoic acids may lead to overproduction of prostaglandins with pro-inflammatory properties in humans. It is aknowledged that increasing the dietary intake of arachidonic acid or its precursor gamma-linolenic acid can lead to an increase in inflammation (Innes & Calder, 2018).

Briefly, for the first time, our results proved that after the addition of AyuFertin, the fatty acid composition of buffalo milk remained unchanged despite the variation in the amount of capric, lauric, stearic and 11-hexadecanoic fatty acids.

CONCLUSION

The supplementation of the postpartum buffaloes with the herbal preparation AyuFertn did not influence negatively test-days productivity parameters and the fatty acid composition in the milk of treated animals, but changed the amount of the long-chain (stearic acid and 11-hexadecanoic) and medium-chain (capric and lauric) acids. The trienoic acids from the herbal preparation, were not transferred into the milk of treated animals. The farmers should strictly follow the manufacturer's recommendation for the dose and duration of treatment with AyuFertin to avoid possible negative effects on milk quality, because AyuFertin was able to change the fatty acids amount in the milk of treated buffaloes.

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