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The Effect of Cu^{+2}, Fe^{+2} and Cr^{+3} in Mineral Additives Enriched with Biosorption Process Form on Chosen Parameters of *in vitro* Caecal Fermentation in Laying Hens (Lohmann Brown)

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Abstract: Trace element additives with Cu (II), Fe (III) and Cr (II) were tested *in vitro* in a Hen Caecum Model. The levels of Short-Chain Fatty Acid (SCFA) were estimated in the 4th and 6th h of fermentation. Measurements of hydrogen and methane as fermentation end products were conducted. Any negative influence on the fermentation process were noted. The study observed a higher production of SCFA in the experimental group than in the control group. In the experiment an increase in propionate acid was observed except in the control and Cr (II) groups. It seems to be true that fermentation in experimental groups was more intensive and faster than in the control one. A result of intensive fermentation was a pH decrease in experimental groups but this was the within range value. The lack of a correlation was shown between the level of acetate and methane and hydrogen concentration.

Key words: Poultry, hen, biosorption, caecum, fermentation, organic sorbent, trace elements

INTRODUCTION

Trace element supplementation is currently one of the most important aspects of animal nutrition (Garg *et al*., 2008; Pechova *et al*., 2009; Janeczek *et al*., 2012). Microelement deficiencies cause insufficiencies leading to production decrease both in animals and humans. Traditionally, trace elements are introduced as inorganic salts or organic mineral products (e.g., amino acid chelates, proteinates and polysaccharides). In poultry nutrition, the most popular form of trace element supplementation are inorganic salts. It is well known that in this form microelements have low bioavailability (Fairweather-Tait, 1999; Nicholson *et al*., 1999; Caussy *et al*., 2003). Additionally in the erlier mentioned case a large emission of the trace elements occurred into the environment (Gustafson and Olsson, 2004). On the other hand, the organic forms like helats are relative expensive and can have a negative influence on the alimentary tract (Chowdhury *et al*., 2004). In recent years the concept of the control of trace elements level in animal products like eggs, meat and milk has been the subject of discussion. This concept is known as biofortification (Johns and Eyzaguirre, 2007). It seems that biofortification could potentially be an important instrument to solve the global problem of hidden hunger (Kennedy *et al*., 2003; Zhao *et al*., 2009; Janeczek *et al*., 2012).

Promising but very rarely discussed in the literature, seem to be alternative products in which trace metals can be bound via naturally occurring processes like biosorption (Hossain *et al*., 1998; Mrvcic *et al*., 2007; Dobrzanski *et al*., 2008; Michalak and Chojnacka, 2008; Michalak *et al*., 2009; Chojnacka, 2010). Biosorption is defined as the passive binding and removal process of metal ions or metalloid species by biological material (Hossain *et al*., 1998; Chojnacka, 2007a, b). The mechanism of biosorption is an ion exchange and complex formation process in the donor-acceptor system of

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electrons from functional groups derived from polymers and macromolecules that are elements of the cell wall. The formation of complexes involves organic compounds containing atoms with free electron pairs, a coordination bond that is formed by oxygen, nitrogen and sulfur (Janeczek *et al*., 2012). It is well known that ion exchange is the dominant biosorption mechanism (Crist *et al*., 1981; Schiewer and Volesky, 1995; Kadukova and Vircikova, 2005). The natural biomass contains light metal ions, e.g., K^+, Na^+, Ca^{2+} and Mg^{2+} that are bound to the functional groups on the cell wall. In the biosorption process these ions are exchanged for other metal ions and their concentration in the solution after biosorption is higher than before the process. At the moment light metal ions and protons are released their binding sites are taken by the microelement ions from the solution (Schiewer and Volesky, 1995; Davis *et al*., 2003). Metal ion binding to the cell wall because of physical adsorption occurs due to electrostatic interactions and Van der Walas forces. In the reaction of metal ions with the components of the cell wall extracellular precipitation may also occur (Veglio and Beolchini, 1997). The main factor responsible for the biosorption is cell wall composition.

The results of the use of biomass enriched with biosorption processes are interesting. In Cu (II) and Fe (II) supplementation as a source of micronutrients in laying hen diet, the following results were observed: increased shell strength lower breakage, higher egg mass lower egg white mass lower parameter L form CIE Color Model of yolk was and higher parameter a eggs fortified with Fe, Zn and Mn were obtained. Eggs from the experimental group were richer in all the examined micronutrients and macronutrients when compared to eggs from the control group (Zielinska, 2010). The preliminary results on the supplements obtained using this method demonstrated that microelements in that form are widely available. Promising results were obtained in the studies on laying hens where using a diet supplement containing Cr^{+3} obtained via biosorption resulted in a considerable increase in the concentration of that element in egg shells was demonstrated (Chojnacka, 2007a; Michalak *et al*., 2009). The fermentation processes in the large intestine are of a high significance in poultry. The fermentation is led by a range of microorganisms. Dietary manipulations of animals results in changes in intestinal flora. The products of fermentation are Short Chain Fatty Acid (SCFA) which may provide 30-40% of metabolic energy (Engelhardt, 1995; Tsukahara and Ushida, 2000; Donalson *et al*., 2008; Mista *et al*., 2011). It is already known that a correlation exists between microorganism populations and SCFA production (Marounek *et al*., 1996; Van der Wielen *et al*., 2000; Donalson *et al*., 2008). The influence of the new mineral feed additives based on biomass that is enriched with microelements in biosorption on animal health including digestive tract status is completely unknown. In poultry, caecal fermentation has decided importance for metabolic energy production and animal health. The biosorbents affecting production of caecal metabolites are not known, yet. The aim of this study was to characterize the influence of microelement supplementation on fermentation parameters (SCFA, methane and hydrogen) in the caecum of hens during *in vitro* investigation.

MATERIALS AND METHODS

Methods of manufacturing diet supplements with microelements: Soybean meal (Vetos, Zebowice near Jawor) was enriched with microelement ions Cu (II), Fe (II) and Cr (III) via biosorption using inorganic salts allowed by law as feed additives: $CuSO_4.5H_2O$, $FeCl_2.4H_2O$, $Cr(NO_3)_3.9H_2O$ (POCh, Gliwice). Biosorption was conducted using a column reactor with a bed of a volume of 0.1 dm^3. Water of pH 5.0 demineralised using 0.1M NaOH/HCl (POCh, Gliwice) was applied in the process. The reaction of water was controlled using a Mettler-Toledo pH-meter (Seven Multi, Switzerland) equipped with an InLab413 electrode with temperature compensation. The biosorption process was conducted at a temperature of 20°C to bed saturation controlling the concentration of solution flowing out of the column. The enriched biomass was air dried for 48 h.

The elemental composition of the samples collected was determined using an ICP-OES plasma spectrometer (Varian Vista-MPX; Varian, PaloALto, USA) in the Chemical Laboratory of Multi-elemental Analysis at Wroclaw University of Technology, accredited by ILAC-MRA and Polish Accreditation Center (PCA) (No. AB 696).

The Control group (C) and three experimental groups were distinguished (Table 1). The availability of a given element from biological preparations was examined in the three groups (Fe, Cr and Cu). The biological preparations were added separately to the prepared feed mixtures (NJT-214, Tasomix®).

The composition of mixtures was established so that it did not contain an addition of a given microelement

Table 1: Content of microelements in non enriched soybean meal before and after biosorption (N = 3)

		Microelement content in soybean meal before and after enrichment (mg g^{-1})	
Microelement	Groups	Non enriched soybean meal	Enriched soybean meal
Fe (II)	Fe	0.235±0.015	16.337±0.228
Cr (III)	Cr	0.022±0.002	20.588±0.212
Cu (II)	Cu	0.065±0.004	15.690±0.370

$\bar{X}\pm SD$ shows the ±values

added in the form of biological preparation (groups Fe, Cr, Cu) and the need for other microelements was fulfilled using inorganic salts. The other components of feed mixtures were the same for all the groups. The requirement for a given microelement was fulfilled 100% by the biological preparation in the experimental groups. The availability of each of the preparations was examined against the control mixture which was a feed mixture containing all microelements in the form of inorganic salts.

In each experimental group, 0.530 g of preparation with Cu (II), 2.755 g of preparation with Fe (II), 4.259 g of preparation with Cr (III) was added to 1 kg of the feed (Table 2). Laying hens microelement requirement:

- Cr: 2 mg kg^{-1}
- Fe: 45 mg kg^{-1}
- Cu: 8 mg kg^{-1}

Animals: The research material consisted of the amount of caecum collected from 50 hens from Hy-Line Brown line, aged 22 weeks. The animals were maintained in a furnished Battery Cage System under controlled microclimatic conditions. The experiments were carried out according to Local Bioethic Committee Permission No. 129/2010.

The obtained mixtures (Table 2) were used as an addition for the incubated content in the group C-control group the complete mixture, group feed with an addition of iron in an organic form group Cr-feed with chromium addition, Cu-group feed with copper addition.

***In vitro* caecal fermentation:** The caecum was collected during dissection and the ingesta was obtained from it.After mixing the ingesta collected from each animal, 5 g of it was divided into serum bottles of a volume of 125 mL (Sigma-Aldrich®). Next, 40 mL of a suitable buffer of pH 7.3 was added to each bottle in order to dilute the ingesta (Janssen *et al*., 2009). The samples were subjected to incubation after addition to 1 g of supplement at a temperature of 39°C. The anaerobic conditions in the bottle were obtained after CO_2 passing. The bottles were tightly closed using a crowner (Restek) with rubber plugs and aluminum caps (Sigma-Aldrich®). The samples placed in the bottles were subjected to 4 and 6 h fermentation *in vitro* in a shaker with water bath. About 48 samples were conducted in total 6 with each feed mixture in particular fermentation hours.

Analysis of collected fermentation products: After incubation, the gas contained in the serum bottles was subjected to analysis in order to examine bacteria activity in the ingesta. The analysis of the produced hydrogen and methane was conducted using gas chromatography on a gas chromatograph (Agilent Technologies 7890A GC System) with TCD and FID detector. After collection of gas samples, measurement of pH of the ingesta was performed and then the sample was centrifuged and 4 M formic acid was added (0.1, 2 mL of solution) in order to inhibit the fermentation processes. The samples of liquid ingesta were subjected to analysis using a gas chromatograph (Agilent Technologies 7890A GC System) with an FID detector in order to determine total SCFA concentration and the percentage contribution of particular acids: acetic, propionic, isobutyric, butyric, isovaleric and valeric.

Statistical analysis: Statistical analyses were done using multi variate analyses of variance using Statistica 9.0 Software (StatSoft Poland, Krakow, Poland). Significant differences were determined using Duncan's test.

RESULTS AND DISCUSSION

The level of active acidity in the caecum content of the hens (Table 3) with the application of iron as a

Table 2: Chemical components of layer ration substrates for *in vitro* fermentation, control group (Tasomix®)

	Groups			
Ingredient content of diets	C	Fe	Cr	Cu
Ground corn (percentage of DM)	29.99	29.99	29.99	29.99
Triticale (percentage of DM)	15.00	18.00	15.00	18.00
Soybean meal (percentage of DM)	13.70	12.10	13.70	12.10
Wheat (percentage of DM)	12.00	10.60	12.00	10.60
Chalk (percentage of DM)	8.440	8.440	8.440	8.440
Decoction wheat-corn (percentage of DM)	6.000	6.000	6.000	6.000
Pszenmix (percentage of DM)	4.500	4.400	4.500	4.400
Sunflower meal (percentage of DM)	4.200	4.300	4.200	4.300
Fats (percentage of DM)	2.300	2.400	2.300	2.400
MPU 2% Nioska St.Tow. (percentage of DM)	2.000	2.000 (no Fe)	2.000	2.000 (no Cu)
Dried full blood (percentage of DM)	1.800	1.700	1.800	1.700
Mycofix select (percentage of DM)	0.050	0.050	0.050	0.050
L-Lysine (percentage of DM)	0.020	0.020	0.020	0.020
Biosorbent addition for 1 kg of feed (g)	-	4.259 Fe (II)	4.259 Cr (III)	0.530 Cu (II)
Lucantin pigment-red	-	-	-	-

Table 3: Production of short-chain fatty acids and levels of the active acidity of the caecal contents of chickens of different mix forage and fermentation time *in vitro*

Groups	Fermentation time (h)	Statistical symbol	pH	SCFA mmol kg^{-1} caecal	Acetate	Propionate	Izobutyrate	Butyrate	Izovalerate	Valerate
					mmol kg^{-1} caecal (%)					
C	4	$\bar{X}$	6.79^{A}	135.28^{Bbc}	51.50	30.81	2.56^{B}	12.10	1.36^{ab}	1.54^{ab}
		SD	0.10	28.66	2.49	2.47	0.53	2.35	0.36	0.70
	6	$\bar{X}$	6.78^{A}	158.43^{ab}	52.20	29.68	3.23^{B}	11.33^{c}	1.60^{ac}	1.92^{bc}
		SD	0.05	19.74	2.62	1.57	0.67	1.85	0.52	0.33
Fe	4	$\bar{X}$	7.05^{B}	134.97^{Bbc}	49.43	31.57	3.05^{Ba}	12.50	1.46^{c}	1.93^{bc}
		SD	0.46	5.43	2.53	2.31	0.43	2.45	0.45	0.07
	6	$\bar{X}$	6.76^{A}	200.80^{ac}	51.11	33.15^{b}	3.11^{Ba}	10.00^{Aa}	1.21	1.39^{Aa}
		SD	0.36	67.09	8.74	8.57	0.68	1.22	0.46	0.24
Cr	4	$\bar{X}$	7.32^{B}	222.36	46.81	34.05	3.65^{B}	11.84^{bc}	1.56^{bc}	2.05^{bc}
		SD	0.44	104.28	4.91	5.93	2.28	3.41	1.04	0.31
	6	$\bar{X}$	7.20^{B}	223.95^{Ac}	44.64	30.53^{b}	6.75^{A}	8.80^{bc}	7.74	1.51^{Bc}
		SD	0.27	98.15	6.82	1.40	4.68	6.66	11.16	1.06
Cu	4	$\bar{X}$	6.79^{A}	136.62^{Bbc}	51.88	28.22^{a}	2.15^{Bb}	14.06^{Bab}	1.22	2.02^{bc}
		SD	0.02	25.85	1.06	0.61	0.29	0.20	0.36	0.50
	6	$\bar{X}$	6.67^{A}	205.61^{A}	52.05	29.49	2.89^{B}	12.50^{bc}	1.27	1.74
		SD	0.03	13.95	1.90	1.57	0.13	0.56	0.21	0.04

^{a,b}Statistically significant differences ($p \leq 0.05$); ^{A,B}Highly statistically significant differences ($p \leq 0.01$)

mineral supplement between 4th and 6th h of fermentation was subject to decrease ($p \leq 0.01$) (of about 0.3 of pH value). The above phenomenon was also observed in other experimental groups however, a decrease between fermentation hours was considerably lower and was from 0.1-0.12 pH. At the 4th h of fermentation, a higher ($p \leq 0.01$) pH content in hens ingesta was observed in the samples after iron and chromium addition (Fe 4 h and Cr 4 h) when compared to the samples with copper addition and the control group (C 4 h and Cu 4 h). At the 6 h of fermentation, the pH value of ingesta after chromium addition (Cr 6 h) was on a higher ($p \leq 0.01$) level when compared to the other groups (C 6 h, Fe 6 h and Cu 6 h).

The lowest level of volatile fatty acids (134.97 mmol kg^{-1}) in hen caecum was noted at the 4th h of fermentation with iron addition (Fe 4 h). The highest in turn (223.95 mmol kg^{-1}) was observed at the 6th h of fermentation when using chromium addition (Cr 6 h). At the 4th h of fermentation of caecum content with the addition of a mineral supplement in the form of chromium (Cr 4 h), an increase in SCFA content of 87.08 mmol kg^{-1} was observed when compared to the control group (C 4 h). After 6 h fermentation of hen ingesta, the supplements used led to an increase in LKT production. An increased ($p \leq 0.05$) general level of acids of 65.52 mmol kg^{-1} when compared to the control group (C 6 h) was noted with chromium application (Cr 6 h). An increase in SCFA production up to 30% was noted at the 6th h of fermentation of caecum content between the group with iron and copper addition (Fe 6 h, Cu 6 h) and the control group (C 6 h).

In *in vitro* conditions at the 6th h of fermentation of caecum content of the hens, the applied mineral supplements caused a decrease in the percentage contribution (mmol kg^{-1} %) of acetic acid when compared to the control group. The lowest level of acetic acid was noted at the 4th and 6th h of fermentation using chromium addition (46.81 and 44.64% mmol kg^{-1}, respectively). In the case of chromium addition, a decrease in acetic acid contribution was observed at the 6th h of fermentation when compared to the 4th h. The reverse tendency was noted in the other experimental groups. A decrease in propionic acid production by caecal bacteria at the 6th h of fermentation was noted in the control group and in the group with chromium addition when compared to the production at the 4th h of fermentation. The reverse relationship was noted in the other experimental groups.

An addition of iron and chromium led to an increase in propionic acid production in the examined caecum content while copper addition caused a decrease in that acid contribution when compared to the control group irrespective of the fermentation time.

At the 4th h of fermentation, a lower contribution of isobutyl acid was noted with a concurrent increase in the level of butyl acid when compared to the 6th h of fermentation irrespective of the feed additive used.

At the 4th h of fermentation, a lower level of isobutyl acid in caecum content was observed after an addition of copper (Cu 4 h) when compared to the control group (C 4 h). The reverse tendency was noted in the other experimental groups. At the 6th h of fermentation, the content of butyl acid in caecum content after copper addition (Cu 6 h) was at a higher level when compared to the control group (C 4 h). The reverse tendency was noted in the other experimental groups.

A lower ($p \leq 0.05$) level of butyl acid (even by 20%) was noted in the caecum content after iron addition at the 6th h of fermentation (Fe 6 h) when compared to the experimental group with an addition of copper and chromium (Cu 6 h, Cr 6 h).

At the 4th h of fermentation iron (Fe 4 h) influenced a higher ($p \leq 0.05$) contribution of isovaleric acid when compared to the ingesta of the control group (C 4 h).

An increase in the level of valeric acid was observed at the 4th h of caecum content fermentation with mineral supplement addition. At the 6th h of fermentation the contribution of valeric acid after iron application (Fe 6 h) was lower when compared to the control group (C 6 h; $p \leq 0.01$) and in relation to the group where chromium was applied (Cr 6 h; $p \leq 0.05$).

Hydrogen production at the 4th h of fermentation (Fig. 1) was at the highest level (0.33 mmol kg^{-1}) in the control group while lowest with iron application (0.18 mmol kg^{-1}). Both in the control group and in the groups where an addition of chromium and copper was used, an increase (even of about 54%-Cr) in hydrogen production was noted between the 4th and 6th h of fermentation. An increase in methane production of 47.65 and 13.38% in the control group and Cu group, respectively (Fig. 2) was observed between the 4th and

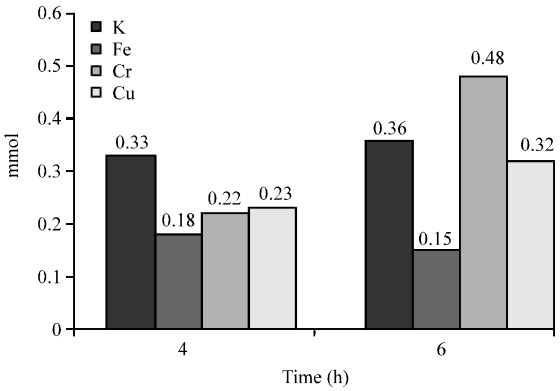

Fig. 1: Concentration of hydrogen (mmol kg^{-1} caecal content)

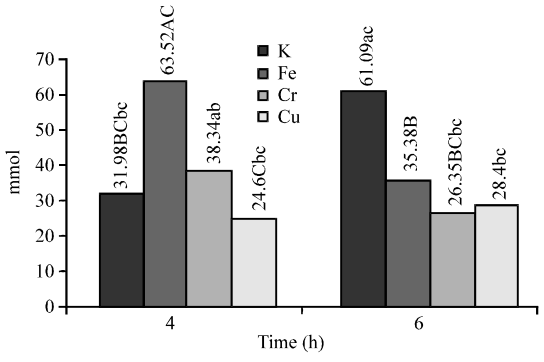

Fig. 2: Concentration of methane (mmol kg^{-1} caecal content). [a,b]Statistically significant differences ($p \leq 0.05$); [A,B]highly statistically significant differences ($p \leq 0.01$)

6th h of fermentation. The reverse tendency was noted in the other experimental groups. At the 6th h of fermentation, higher ($p \leq 0.05$) methane production was noted in the control group (C 6 h) when compared to caecum content production with chromium (Cr 6 h) and copper (Cu 6 h) application. The methane content in the samples collected at the 4th h of fermentation was at a higher level with iron application (Fe 4 h) when compared to the other groups.

In the caecum of hens, microbial fermentation is a source of SCFA. This process can be modified by a great number of factors (Marounek *et al*., 1999; Shanmugavelu *et al*., 2006). The SCFA play an important role in animal metabolism and they have also a significant importance in physiological equivalence in caecum as a biotope. Too low production of SCFA favors intestinal mucous colonization by pathogens like *Salmonella* sp. (Barnes *et al*., 1979; Corrier *et al*., 1995; Kubena *et al*., 2001). It seems that the microflora of caecum and fermentation profile are important aspects of animal health.

The pH level in caecum should be of 5.65-7.8 (Jozefiak *et al*., 2004). It has been proved that lower pH (pH-5.8) has a negative influence on acetate and propionate acid production (Meimandipour *et al*., 2010). In the investigation pH level was in the physiological range of value but a decrease in its level was observed between 4th and 6th h. This pH decrease was higher in the experimental than control groups. Probably, this effect was caused by an increase in fermentation product concentration (Mista *et al*., 2011). It should be taken into consideration that feed additives were generated on the basis of the soy pellets which contain carbohydrates and its role in pH decrease in caecum is well known (Berggren *et al*., 1993). The results presented in this research do not indicate that low pH changes influence the qualitative composition of SCFA.

Plant polysaccharides are used in SCFA and gases like ammonia, carbooxydate, methane and hydrogen in caecal production (Engelhardt, 1995; Tsukahara and Ushida, 2000; Donalson *et al*., 2008). In the investigation the influence of microelement additives on SCFA increase was presented. The mean value of SCFA concentration/kg caecal content in a hen equals 107-151 mmol kg^{-1} (Jozefiak *et al*., 2004). According to Tsukahara and Ushida (2000) the SCFA production in caecum is time dependent, i.e., 32.1-35.2 mmol kg^{-1} in the 1st h, 128.4-140.8 mmol kg^{-1} in the 4th h and 192.6-211.2 mmol kg^{-1} in the 6th h. In the investigation, the level of SCFA in the 6th h of fermentation was higher than in the control group but not in a statistically important manner except fermentation with Cr. It should be pointed out that the level of SCFA was higher in the group with Cr than in other experimental and control groups after 4 h of fermentation. The results indicate that the effect of biosorbent additive introduction was fermentation increase (pH level decrease and SCFA concentration level increase).

SCFA production is an important parameter of microbial fermentation in caecum. It is already known that caecal SCFA are a significant factor in preventing caecal Salmonella colonization (Barnes *et al*., 1979). Especially important is the propionate acid level because of the proven negative correlation between its caecal concentration and Salmonella colonization in young chickens (Saengkerdsub *et al*., 2006). The relationship between propionate concentration and some microbial agents, e.g., *Salmonella typhimurium* and Enterobacteriaceae has been shown (Van der Wielen *et al*., 2000). It was proved that the optimal proportion between acetate, propionate and butyrate acids should equal 3-5:2:1 (Marounek and Rada, 1998; Saengkerdsub *et al*., 2006; Donalson *et al*., 2008; Meimandipour *et al*., 2010). Some other researchers suggest optimal concentrations of acetate, propionate, izobutyrate, butyrate, izovalerate and valerate of 48-65, 12-32, 0.3-3, 6-14, 2-3 and 3-10% in caecum (Marounek and Rada 1998; Saengkerdsub *et al*., 2006; Donalson *et al*., 2008). In the investigation similar proportions were observed. In the case of additional Fe and Cu, the concentration of propionate increased in the 6th h of fermentation but in Cr decreased.

The production of gas as an end product of caecal fermentation provides a reproducible *in vitro* method for determining the potential effects of feed additives on gut flora (Saengkerdsub *et al*., 2006). Guo *et al*. (2003) results proved gut gas production increased after polysaccharides intake (e.g., yeast). Methane production level is an indicator of carbohydrate fermentation and it has a positive correlation with SCFA production. The reduction of acetate production as a result of methanogenesis can be energy-efficient (Varadyova *et al*., 2000). High levels of hydrogen can be the result of its accumulation during intensive reduction processes in acetate production (Marounek and Rada, 1998). In the investigation significant changes of acetate concentration were not proved. The lack of correlation between levels of acetate and methane and hydrogen concentration was stated. However, a higher level of hydrogen concentration after 6 h of fermentation in group with Cr addition was observed.

It is known that hydrogen and methane production per hour of fermentation should be of 0.07 and 11.4 mmol kg^{-1} of gut content (Saengkerdsub *et al*., 2006). In 4 months old chickens the ratio between hydrogen and methane is 1:9 (Marounek and Rada, 1998). In this study the erlier mentioned ratio was similar. It seems to be true that fermentation in the group with Fe (III) additive was more intensive and faster than in other groups (Fig. 2).

The preliminary results of the investigations suggest that the introduction of trace element additives generated with biosorption do not have a negative influence on microbial fermentation in caecum of laying hens.

CONCLUSION

According to the analyzed parameters, it seems that the Cu (II) addition in the new form had the most positive effect on fermentation. Because of the positive accumulation of Fe (III), Cu (II) in technological processes high bioavailability and low emission to the environment, biosorbents can be an alternative source of trace elements for animal feeding.

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