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The Effect of Dietary Zinc (II) Chelate and Zinc (II) Enriched Soybean Meal on Selected Parameters of *In vitro* Caecal Fermentation of Laying Hens

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Abstract: Soybean meal was enriched with Zn (II) ions via biosorption using inorganic salt ($ZnSO_4.7H_2O$). Biosorption was conducted using a column reactor with a bed of a volume of 0.1 dm^3. 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 control group (C) and two experimental groups (I and II) were distinguished. The availability of zinc from the preparation obtained via biosorption method was examined in the first group (I) while the availability of zinc in a from organic chelate was assessed in the second group (II). The caecum was collected during dissection and the ingesta was obtained from it. 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 were conducted using gas chromatography method on a gas chromatograph. 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. The results analysis proved no negative influence of soybean meal enriched with zinc using biosorption method on *in vitro* fermentation process in caecum.

Key words: Poultry, hen, biosorption, caecum, fermentation, organic sorbets, zinc

INTRODUCTION

Micronutrient malnutrition is a serious health problem worldwide, affecting >3 billion people (Kennedy *et al*., 2003; Welch and Graham, 2004). The most prevalent deficiencies of micronutrients are Fe, Zn, vitamin A and I which occur particularly among women and children in the developing countries (WHO, 2002). It has been estimated that Fe and Zn deficiencies each affect about one-third of the world's population (Hotz and Brown, 2004; WHO, 2002). It seems that an integration of micronutrient-rich foods such as vegetables, fruits and animal products into the diets is most practical and sustainable way to alleviate micronutrients deficiency (Prakash, 2009). Having above in mind, the concept of so called biofortified food has been developed. Such a food would contain a suitable level of microelements. Biofortification aims to enhance micronutrient concentrations and/or bioavailability in plant and animal food products. In case of animal origin products this would be obtained via suitable supplementation (Janeczek *et al*., 2012). Zinc is a trace element essential for proper growth and development of hens (Bou *et al*., 2005; Park *et al*., 2004; Sahin *et al*., 2006). The most widely used products for zinc supplementation are inorganically (zinc sulfate and zinc oxide) and recently, organically bound zinc supplements used in animal diets (Sobhanirad *et al*., 2010). Zinc salts are easily dissolved in water and form radicals what leads to fats and vitamins degradation. Zinc oxide in turn, does not influence

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negatively fats and vitamins but is to a smaller degree available to the poultry (Park *et al*., 2004). Microelements administered to hens in a form of organic compounds are available in a better manner when compared to their inorganic forms (Bao *et al*., 2010).

However, it is well known that microelements in an inorganic form possess very low bioavailability and are of transit character, they pass through the alimentary canal of an animal and are absorbed only to a very low extent. The majority is excreted in the form of droppings which cause an serious environmental problem. For this reason, there is a need to elaborate new mineral feed additives. Recently, a new group of biological feed additives was elaborated in which biomass is enriched via biosorption (Chojnacka, 2007; Johns and Eyzaguirre, 2007; Michalak and Chojnacka, 2009).

Biosorption is a term that can be defined as the passive binding/removal of metal ions or metaloid species, compounds and particulates from solution by biological material. The postulated mechanism of biosorption is an ion exchange and complex formation process in donor-acceptor system of electrons from functional groups derived from polymers and macromolecules that are elements of the cell wall. The formation of complexes involve organic compounds containing atoms with free electron pairs, a coordination bonds which are formed by oxygen, nitrogen and sulfur (Janeczek *et al*., 2012). Preliminary results of the studies on feed mixtures enriched with Cu (II), Zn (II), Co (II), Mn (II) and Cr (III) using biosorption method demonstrated considerably higher level of these elements accumulation against control groups supplemented using microelements in a form of inorganic salts in egg yolk, egg white and blood of hens. The egg weight and eggshell thickness parameters in groups fed with the diet containing biosorbents were higher than in the control group (Michalak *et al*., 2011).

Thus, while an influence of feed additives obtained via biosorption on animals origin products in laying hens is known, their influence on transformations occurring in large intestine which are of an essential meaning for health and thus productivity of the animals has not been recognized.

The aim of the study presented is to compare selected parameters of fermentation transformations in *in vitro* conditions using feed additive obtained via biosorption process and in a form of chelate.

MATERIALS AND METHODS

Methods of manufacturing diet supplements with microelements: Soybean meal (Vetos, Zebowice near Jawor, Poland) was enriched with Zn (II) ions via biosorption using inorganic salt ($ZnSO_4 \cdot 7H_2O$) allowed by law as feed additives (POCh, Gliwice, Poland). 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, Poland) 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.

Table 1: Content of zinc in non enriched soybean meal and after biosorption process. Content of zinc in soybean meal before and after enrichment (mg g^{-1})

Non enriched soybean meal ($\bar{x} \pm SD$, N = 3)	Enriched soybean meal ($\bar{x} \pm SD$, N = 3)
0.054±0.005	14.088±0.403

The level of zinc in 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) Table 1.

The control group (C) and two experimental groups (I and II) were distinguished. The availability of zinc from the preparation obtained via biosorption method was examined in the group (I) while the availability of zinc in a from organic chelate was assessed in the second group (II). The biological preparation and chelate were added separately to the prepared feed mixtures (NJT-214, Tasomix). The composition of mixtures was established so that it did not contain zinc addition, an a requirement for the other microelements was fulfilled using inorganic salts. The other components of feed mixtures were the same for all the Table 2. The requirement for zinc in I group was fulfilled 100% by the obtained biological preparation. In II group, the requirement for zinc was fulfilled 100% by zinc chelate (Glystar Forte, Agsol). The availability of each of zinc preparations (I group) and chelate (II group) was examined against the control mixture which was a feed mixture containing all microelements in a form of inorganic salts.

An amount of 4.258 g of the preparation with Zn (II) was added to the feed in I group and 0.375 g of organic chelate with zinc in II group.

Animals: The research material consisted of the amount of caecum collected from 38 hens of Hy-Line Brown line, aged 22 weeks. The animals were maintained in a furnished battery cage system under controlled microclimatic conditions.

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

	Groups		
Ingredient content of diets	C	I	II
Ground corn (% of DM)	29.99	29.99	29.99
Triticale (% of DM)	15.00	18.00	18.00
Soybean meal (% of DM)	13.70	12.10	12.10
Wheat (% of DM)	12.00	10.60	10.60
Chalk (% of DM)	8.44	8.44	8.44
Decoction wheat-corn (% of DM)	6.00	6.00	6.00
Pszenmix (% of DM)	4.50	4.40	4.40
Sunflower meal (% of DM)	4.20	4.30	4.30
Fats (% of DM)	2.30	2.40	2.40
MPU 2% Nioska St.Tow. (% of DM)	2.00	2.00 (no Zn)	2.00 (no Zn)
Dried full blood (% of DM)	1.80	1.70	1.70
Mycofix Select (% of DM)	0.05	0.05	0.05
L-Lysine (% of DM)	0.02	0.02	0.02
Addition for 1 kg of feed (g)	-	4.259 Zn (II)	0.375 Zn (II)-chelate
Lucantin pigment-red	-	-	-

The obtained mixtures (Table 2) were used as an addition to incubated content, in group C: control group, the complete mixture, I group: the mixture with zinc introduced via biosorption method, II group: mixture with zinc in a form of chelate. The research gained an acceptance of the bioethical commission 129/2010.

***In vitro* caecal fermentation:** The caecum was collected during dissection and the ingesta was obtained from it. After mixing, 5 g of the ingesta 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 an addition of 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 36 samples were collected in total, 6 with each feed mixture in particular fermentation hours.

Analysis of selected 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 were conducted using gas chromatography method on a gas chromatograph (Agilent Technologies 7890A GC System) with TCD and FID detectors. After collection of gas samples, measurement of pH of the ingesta was performed and then the samples were centrifuged and 4 M formic acid was added (0.1 mL/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 multivariate analyses of variance using Statistica 9.0 Software (StatSoft Poland, Krakow, Poland). Significant differences were determined using Duncan's test.

RESULTS

The level of active acidity in the caecum content of the hens between the 4th and 6th h of fermentation (Table 3) was subject to a slight decrease in the control group. The reverse tendency was noted in the other groups. An application of the preparation obtained via biosorption method (I) influenced an increase in pH value of caecum content when compared to the control group (C) and the group in which zinc was used in a form of chelate (II), irrespective of fermentation time.

The lowest level (101.72 mmol kg^{-1}) of volatile fatty acids (SCFA) in hen caecum content was noted in the 4th h of fermentation with an addition of the preparation after biosorption (I 4 h).

An application of zinc in a form of organic chelate (II) in *in vitro* fermentation of hen caecum content caused an increased by 14.43 mmol kg^{-1} SCFA production in the 4th h and by 9.98 mmol kg^{-1} in the 6th h when compared to the control group. In turn, an application of zinc in preparation after biosorption (I) resulted in a decreased level of volatile fatty acids.

In an *in vitro* conditions, in the 6th h of fermentation of hen caecum content, the application of addition of zinc introduced via biosorption method (I 6 h) caused a decrease ($p = 0.05$) in the percentage contribution (% mmol kg^{-1}) of acetic acid when compared to the Control group (C 6 h). Similar relationship was observed in the 4th h of fermentation. An introduced zinc supplement, irrespective of chemical form (I and II), influenced a decrease in the percentage contribution of acetic acid content and an increase in the level of isobutyl, butyl, isovaleric and valeric acids in the 6th h of hen caecum fermentation when compared to the 4th h of fermentation. The reverse relationship was noted in the control group for acetic, butyl and isovaleric acids.

In the 4th h of hen caecum content fermentation, lower ($p \leq 0.01$) propionic acid concentration was noted in fermentation products with an application of zinc in a form of organic chelate (II 4 h) when compared to zinc introduced via biosorption (I 4 h).

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	$\bar{x}$	6.80	134.29	51.84^{b}	31.01	2.46	11.11	1.43^{b}	1.30^{b}
		SD	0.05	29.77	2.38	3.06	0.63	2.38	0.45	0.76
	6	$\bar{x}$	6.78	165.25^{b}	52.75^{b}	29.38^{b}	3.16	10.98	1.42	1.75
		SD	0.07	21.26	2.06	1.94	0.71	2.16	0.34	0.43
I	4	$\bar{x}$	6.90	101.72^{a}	49.69	33.89^{Bc}	2.67	10.54^{a}	1.40^{b}	1.77
		SD	0.20	6.63	3.23	0.90	0.75	3.03	0.16	0.40
	6	$\bar{x}$	6.93	136.78	46.44^{Aa}	33.54	3.03	12.96	2.01^{Aa}	1.98
		SD	0.26	18.71	6.00	4.86	0.99	0.77	0.58	0.22
II	4	$\bar{x}$	6.75	148.72	53.62^{B}	28.42^{Aac}	2.57	12.02	1.46^{B}	1.85
		SD	0.10	36.40	2.74	2.06	0.39	1.43	0.51	0.31
	6	$\bar{x}$	6.81	155.27^{b}	50.35	29.84^{ab}	3.05	12.74^{b}	1.62	2.12^{a}
		SD	0.13	69.82	2.37	1.12	0.27	1.40	0.38	0.55

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

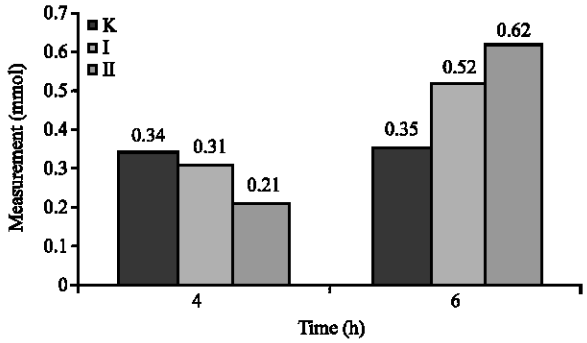

Fig. 1: Concentration of hydrogen (mmol kg^{-1} cecal content)

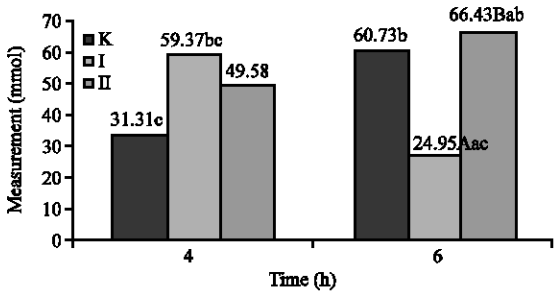

Fig. 2: Concentration of methane (mmol kg $^{-1}$ cecal content. ^{a,b}Statistically significant differences ($p \leq 0.05$); ^{A,B}Highly statistically significant differences ($p \leq 0.01$)

Higher contribution of propionic acid was noted in caecum content of hens with an application of zinc addition after biosorption (I), irrespective of fermentation hour when compared to the control group (C).

The levels of butyl, isovaleric and valeric acids were higher in the products of hens caecum content fermentation after zinc addition in a form of organic chelate (II) when compared to the control group (C). Moreover, the percentage contribution of valeric acid was higher in the group where zinc introduced via biosorption method was applied, in relation to the control group (C).

No unequivocal influence of the applied supplements on the change in the level of analyzed volatile fatty acids was noted in the other groups.

Hydrogen production in the 4th h of fermentation (Fig. 1) was on the highest level (0.34 mmol kg^{-1}) in the control group while on the lowest one in the case of zinc application in a form of chelate (0.21 mmol kg^{-1}). The reverse tendency was observed in the 6th h of fermentation of hen caecum content. Hydrogen synthesis was maintained on the highest level in the samples where zinc addition in a form of chelate was applied and it was the lowest in the control group. Both in the control group (C) and in the groups where an addition of zinc was used (I and II), an increase (even by about 66%-II group) in hydrogen production was noted between the 4th and 6th h of fermentation. An increased by 29.42% ($p \leq 0.05$) methane production was noted in the control group (C) (Fig. 2) between the 4th and 6th h of fermentation. Similar relationship was noted in the group where zinc chelate (II) was used. In I group in turn with an application of zinc introduced via biosorption, lower ($p \leq 0.05$) methane production was observed in the 6th h of fermentation (I 6 h) when compared to the 4th h of fermentation (I 4 h).

DISCUSSION

The pH value of the examined samples ranged on a level from 6.75-6.93. Jozefiak *et al.* (2004) and Lan *et al.* (2006) obtained in their research similar values of active acidity in hens caecum content. According to Ricke *et al.* (2004) the pH value of hens caecum content decreases with lowered calcium content (800 mg kg^{-1}) in feed and increased zinc content (110 mg kg^{-1}). Zinc in an inorganic form was used in feed in the control group in the examined samples while in the experimental groups it was applied in

a form of organic chelate and in preparation after biosorption (60 mg kg^{-1}) and no differences in pH value were noted between the samples obtained.

According to other researchers, SCFA production in caecum in the 1st h of fermentation ranges from 32.1-35.2 mmol kg^{-1} what gives 128.4-140.8 mmol kg^{-1} in the 4th h and from 192.6-211.2 mmol kg^{-1} in the 6th h (Tsukahara and Ushida, 2000). The level of volatile fatty acids in the examined samples in the control group was on a similar level. Zinc addition insignificantly lowered volatile fatty acids production, probably because the form of zinc was more bioavailable in the experimental groups. Feed additives used in the fermentation process were manufactured based on, e.g., soybean meal which contained oligosaccharides. They influence an increase in VFA level (Lan *et al*., 2006). Zinc exhibits bactericidal activity and fermentation process in caecum depends on bacterial flora (Marounek and Rada, 1998; Van der Wielen *et al*., 2000; Ricke *et al*., 2004; Bou *et al*., 2005). According to Ricke *et al*. (2004) an overall production of volatile fatty acids in hens caecum content decreases with lowered calcium content (800 mg kg^{-1}) and increased zinc content (110 mg kg^{-1}) in feed.

The ratio of acetic acid level to propionic and butyric ones in the process of fermentation in caecum should be 3-5:2:1 for the adult animals (Marounek and Rada, 1998; Saengkerdsub *et al*., 2006; Donalson *et al*., 2008; Meimandipour *et al*., 2010). In young broilers (11 weeks old) in turn, this ratio is slightly different and reaches 3:2.5:2 (Lan *et al*., 2006).

An excessive amount of zinc in feed dose influences disturbance in these acids ratio and it is then 6.5:2.5:1 (Ricke *et al*., 2004). No negative influence of the applied fodders on the ratio of acetic to propionic and butyl acid was noted in the examined samples and it was 4.5-5:3:1 in the control group and 5-4:2.5-3:1 in the experimental ones. According to other researchers, the contribution of acetic, propionic, isobutyric, butyric, isovaleric and valeric acids in fermentation process of caecum content should be 48-65, 12-32, 0,3-3, 6-14, 2-3 and 3-10%, respectively (Marounek and Rada, 1998; Saengkerdsub *et al*., 2006; Donalson *et al*., 2008). Lower percentage contribution of isovaleric and valeric acids was noted in the examined samples. The percentage contribution of the other acids was similar in the control group and in the group where zinc was used in a form of organic chelate. Higher level of propionic acid was observed in the group where the feed was enriched with zinc via biosorption. The content of this acid is a significant index in an assessment of health status, since there is a relationship between this acid concentration and an amount of inter alia *Salmonella typhimurium* Enterobacteriaceae.

Propionic acid is metabolized in liver and is indirectly involved in citric acid cycle (Engelhardt, 1995). It may be thus supposed that an application of zinc introduced via biosorption in feed and to a lower degree zinc in a form of organic chelate, influences decrease in the level of pathogenic bacteria and an increase in energy obtained during fermentation processes. However, the results obtained require *in vivo* study with an additional assessment of caecum bacterial flora.

Methane level proves mainly an intensity of carbohydrates fermentation and usually increases with an increase in volatile fatty acids production. Acetate genesis reduction in place of methane genesis allows for metabolic energy saving (Varadyova *et al*., 2000). The above relationship was not observed in the samples analyzed.

Hydrogen and methane production per an hour of fermentation should be 0.07 and 11.4 mmol kg^{-1} of caecum content what gives 0.28 and 45.6 and 0.42 and 68.4 mmol kg^{-1} (hydrogen and methane, respectively) in the 4th and 6th h of fermentation (Saengkerdsub *et al*., 2006).

Too high production of methane leads to energy losses while an excessive hydrogen production may be caused by that compound accumulation with an excessive reduction in acetate genesis (Marounek and Rada, 1998; Marounek *et al*., 1999). In the analyzed samples, insignificantly higher production of hydrogen up to 0.34 mmol kg^{-1} in the 4th h of fermentation and up to 0.62 mmol kg^{-1} in the 6th h of fermentation was observed in most of the cases. Methane concentration in the 6th h of fermentation was the lowest in the samples where an additive after biosorption was applied (24.95 mmol kg^{-1}).

CONCLUSION

The study conducted demonstrated that an application of zinc in a form of organic chelate and zinc introduced to the feed via biosorption method does not influence negatively the products of caecum fermentation in hens. Taking into account the level of volatile fatty acids being fermentation products, more profitable influence of feed with an addition of zinc in a form of organic chelate was observed. However, zinc introduced via biosorption resulted in a lower level of hydrogen and methane production what in a consequence may influence limitation of energy losses by the animals. Due to well zinc accumulation during technological processes in feeds, the biosorbents may be applied as an alternate source of that microelement with respect to available on a market inorganic forms for the animals.

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