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Iron and Zinc Availability to Broiler Chicken from Mineral Biofortified Wheat

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Abstract

This study was conducted to investigate the effect of mineral biofortified wheat with fungal phytase addition on iron (Fe) and zinc (Zn) availability in broiler chicken breast muscle and liver, their excretion in feces and digestibility in intestines. Furthermore, possible beneficial effect on broiler performance was studied. 90 day-old Ross 308 broiler chickens were placed in brooder cages and received commercial starter diet from day 1-7 posthatch and micro-mineral deficient starter diet from day 7-11 posthatch. On 11 day posthatch 70 randomly selected chickens were weighed, placed in individual cages and assigned to six experimental diets and one commercial grower diet. Experimental diets consisted of three different biofortified wheats as cereal components (difference is in the level of Fe and Zn in wheat), soybean meal, soybean oil and contained titanium dioxide as an indigestible marker. Three experimental diets were supplemented with recommended levels of fungal phytase. Broilers were weighed again on 19 day posthatch and number was reduced to 49 (7 replicants per diet). Feed intake and excreta output quantification was performed from day 19 until day 22 posthtach. On 22 day posthatch birds were killed by dislocation of their neck and samples of liver and breast muscle were taken. All samples were analysed by ICP-MS. Excreta samples were analysed to determine the excretion and digestibility of the Fe and Zn, and liver and breast muscle samples were analysed in order to detect content of Fe and Zn. No differences were observed for body weight gain, feed intake or feed conversion ratio among all different dietary levels of Fe and Zn. Fecal mineral excretion increased linearly with increased levels of Fe and Zn in biofortified wheat and it was significantly higher ($P < 0.001$) in control diet when compared to experimental diets. Apperent digestibility of Fe increased linearly with increased level of Fe in biofortified wheat, while digestibility of Zn decreased linearly with increased levels of Zn in biofortified wheat. Highest digestibility of Fe was in diet with 220 mg Fe/kg diet and highest digestibility of Zn was in diet with 37 mg Zn/kg diet. Supplementation of fungal phytase had no effect on broiler performance or mineral digestibility in this study.

Key words: iron, zinc, bioavailability, digestibility, phytase, titanium dioxide

Sažetak

Ova studija je provedena u cilju otkrivanje uticaja bio-fortificirane pšenice sa dodatkom fungalne fitaze na dostupnost cinka (Zn) i željeza (Fe) u prsnom mišiću i jetri brojlera, te na njihovu ekskreciju u fecesu i razgradivost u crijevima. Mogučnost pozitivnog efekta na performans brojlera je takođe bio jedan od ciljeva studije. 90 dan starih brojlera rase Ross 308 je postavljeno u kaveze i u prvih sedam dana života su hranjeni komercijalnom starter dijetom, a od 7-11 dana života su hranjeni sa starter dijetom koja je deficitarna u mikro mineralima. 11 dana života, 70 nasumično odabranih pilića je izvagano i postavljeno u individualne kaveze te im je za ishranu dodjeljena jedna od šest eksperimentalnih dijeta ili komercijalna grover dijeta. Eksperimentalne dijete su sadržavale tri različite vrste bio-fortificirane pšenice kao osnovnu komponentu (razlike su bile u razini Fe i Zn u pšenici), sojinu sačmu, sojino ulje i titanium dioksid kao nerazgradivi marker. Tri eksperimentalne dijete su suplementirane sa preporučenim količinama fungalne fitaze. Pilići su ponovo izvagani 19 dana života i broj pilića je smanje na 49 (7 replikanata po dijeti). Kvantitativno mjerenje unosa hrane i ekskrecije je obavljeno od 19 do 22 dana života. 22 dana života pilići su ubijeni metodom dislokacije vrata i uzorci prsnog mišića i jetre su uzeti. Uzorci ekskreta su analizirani u cilju određivanja ekskrecije i razgradivosti Fe i Zn a uzorci prsnog mišića i jetre su analizirani u cilju određivanja sadržaja Fe i Zn u njima. Nije uočena nikakva značajna razlika u težini, unosu hrane i konverziji hrane između različitih Fe i Zn. Mineralna ekskrecija je linearno povečana sa povečanjem razine Fe i Zn u bio-fortificiranoj pšenici i bila je značajno veča ($P < 0.001$) u kontrolnoj dijeti nego u eksperimentalnim dijetama. Razgradivost Fe je linearno povečana sa povečanjem razine Fe u bio-fortificiranoj pšenici, dok je razgradivost Zn linearno smanjena sa povečanjem razine Zn u bio-fortificiranoj pšenici. Največa razgradivost Fe je uočena kod dijete sa 220 mg Fe/kg dijete dok je največa razgradivost cinka uočena kod dijete sa 37 mg Zn/kg dijete. Suplementacija fungalne fitaze nije imala značajan efekat na performans brojlera ili razgradivost mineral u ovoj studiji.

Ključne riječi: željezo, cink, biodostupnost, razgradivost, fitaza, titanium dioksid

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1. Introduction

Iron (Fe) and zinc (Zn) are necessary for almost all living organisms and they play important role in many metabolic processes. Zinc is included in DNA synthesis, cell division, gene expression and also it is key component of many enzymes in human and animal body (Prasad, 1991). Iron is required in synthesis of DNA, RNA and proteins and it is essential for cellular enzymes, such as oxidases, catalases, peroxidases, cytochromes, ribonucleotide reductases: aconitases, and nitric oxide synthases (Lieu et al., 2001). A deficiency of iron and zinc can result in metabolic disorders and compromise the health of the organism (Lopez et al., 2002). Unfortunately there are millions of people in the world who suffer from deficiency of essential metals like iron and zinc. Zinc deficiency occurs most often in the regions where soil is Zn-deficient and studies indicate that nearly half of the world population suffers from zinc deficiency (Cakmak, 2008). On the other hand according to World Health Organization (WHO) iron deficiency is the most common nutritional disorder in the world and it has epidemic proportions.

One of the main reasons for iron and zinc deficiency is due to high phytic acid (myo - inositol 1, 2, 3, 4, 5, 6 - hexakisphosphate) content in feed and fodder fed to animals. Phytic acid is the main phosphorus storage compound in plant seeds and it may constitute for up to 80% of total phosphorus in seed (Lopez et al., 2002). Metallic cations of iron and zinc are linked to the negatively charged phosphate in phytic acid and that bound is making them insoluble and unavailable for absorption (Bohn et al., 2008).

Cakmak (2008) found that application of zinc fertilizers or zinc enriched NPK fertilizers (e.g., agronomic biofortification) can be one of the main solution to combat iron and zinc deficiency. Another opinion was that the bioavailability of iron and zinc consumed can be increased by promoting the intake of enhancers and reducing the impact of phytic acid on intestinal absorption (Lopez et al., 2002).

Bosnia and Herzegovina (BIH), Croatia and Serbia are geographically located in southeastern Europe, in the western part of the Balkan Peninsula. These countries possess extremely heterogenic soils as a result of a great heterogeneity of geological base, climate, vegetation, and paedo-fauna (Manojlovic and Singh, 2012).

Levels of iron and zinc in western Balkan countries soils differ between different regions. In the region of western BIH and western Serbia, levels of iron are very high due to the presence

of ultrabasic rocks and serpentinites (Manojlovic and Singh, 2012). Higher levels of iron and zinc can also be found in northeast Bosnia and they are associated with breakthroughs of tertiary igneous rocks (Midzic and Silajdzic, 2005b). Alijagic and Sajn (2006) reported high levels of iron and zinc in the soils of metallurgic areas in central Bosnia as consequences of ironwork and zinc mines in that area.

On the other hand in the eastern parts of Croatia and the Serbian part of the Pannonian valley soils are iron and zinc deficient (Jug et al., 2008, Manojlovic and Singh, 2012). Deficiency usually happens as a result of high pH values and alkalinity of soils, but it can also be associated with prolonged wet soil conditions or poor drainage, and low soil temperature. Cuvardic et al., (1993) found that the content of available zinc in soil samples taken under field crops located in the regions of Banat and Srem (Serbia) were insufficient for the production of both field crops and vegetables.

Despite the fact that total amount of iron and zinc in some soils in western Balkan is high, there are still problems, because the total amount of elements in soil is not a good indicator of amount that can be taken by plants and enter food chain (Manojlovic and Singh, 2012).

Cengic, (2000) reported that amounts of zinc in investigated feedstuffs for ruminants in the BIH varied according to published values, and that content of zinc in forage and sugar beet pulp could not satisfy dairy cow's demands. Concentration of iron and zinc in the grain among 121 genotypes of maize grown in a field trial in Eastern Croatia varied significantly, and grain concentration of iron and zinc ranges were 11.0 to - 60.7 (Fe) and 11.9 to - 33.2 (Zn) (mg/kg dry weight) (Brkic et al., 2004). Low amounts of available zinc and iron in soil and alfalfa samples were measured in soils with low texture as well as those with high pH-value and content of calcium carbonate in Vojvodina Province (Cuvardic et al., 2006).

Primary deficiency of iron and zinc is rare in western Balkan countries, but secondary deficiency is very frequent. Zinc deficiency is more often in animals, especial young animals and non-ruminants and sometimes it can be asymptomatic. On the other hand iron deficiency is more frequent in humans and in western Balkans it is the most common mineral deficiency. According to the report Zdravstveno stanje stanovnistva i zdravstvena zastita u Federaciji Bosni i Hercegovini, (2012), prevalence of iron deficiency in Bosnia and Herzegovina by categories is:

1. Children from 11 to 59 month: 18%

2. Children from 5 to 15 years of age: 11.5%
3. Woman from 15 to 49 years of age: 22.1%

Prevalence of iron deficiency among children in Croatia is 2.2% for the first grade children and 3.5% for the seventh grade children (Antonic Degac et al., 2002).

Keeping the above facts in view the main objective of the study was to: Assess the availability of Fe and Zn to broiler chickens by feeding them with mineral biofortified wheat coupled with phytase addition to improve the concentration of these elements in chicken meat.

Sub-objectives:

1. Analyze iron and zinc content in feed.

2. Measure retention of iron and zinc by feed intake, digestibility and excretion in feces.

3. Analyze the impact of increased concentration of Fe and Zn in feed on their level in broiler chicken breast muscle and liver.

4. Determine the effect of phytase addition on bioavailability of iron and zinc.

Hypothesis:

(H_O) Increased amount of iron and zinc in feed containing biofortified wheat and enzyme phytase will increase iron and zinc availability and level in chicken breast muscle and liver.

(H_1) Increased amount of iron and zinc in feed containing biofortified wheat and enzyme phytase will not increase iron and zinc availability and level in chicken breast muscle and liver.

2. Literature

2.1. Iron (Fe) and zinc (Zn)

2.1.1. Importance of iron and zinc

Iron (Fe) and zinc (Zn) are essential trace elements and they play important role in many metabolic processes in human and animal organism. Majority of iron is present in the erythrocytes as hemoglobin (molecule that contains one hem group and one protein chain in each of its four units). This structure of hemoglobin will stabilize iron in the ferrous state and allow it to function as oxygen carrier from the lung where it is fully loaded with oxygen to the tissue where is partially unloaded.

The rest of the essential body iron is present in the forms of myoglobin, cytochromes, and iron-containing enzymes such as oxidases, catalases, peroxidases, ribonucleotide reductases, aconitases, and nitric oxide synthases (Boldt, 1999; Conrad et al., 1999; Lieu et al., 2001).

Except his critical role in oxygen transport iron plays important roles in cellular processes including synthesis of DNA, RNA, and proteins, electron transport, cellular respiration and regulation of gene expression (Conrad et al., 1999; Lieu et al., 2001;). Iron also affects cell cycling and differentiation by supporting transcription of certain genes (Boldt, 1999; Lieu et al., 2001) and it maintains cellular iron homeostasis by regulating gene expression at the posttranscriptional level (Haile, 1999; Lieu et al., 2001).

Zinc is one of the metal ions, presented in all body tissues and fluids and it is a structural component of a large number of proteins. It is estimated by researchers that up to 10% of human proteins bind zinc (Andreini et al., 2006). Interactions between the proteins and other macromolecules such as DNA are facilitated by the binding of zinc who stabilizes folded conformations of domains. (Berg and Shi, 1996). Flexible coordination geometry of zinc ions allows zinc binding proteins to shift conformations quickly in order to carry out complex biological reactions (Berg and Shi, 1996; Liu et al., 2012; Wellinghausen et al., 1997).

Bioavailable zinc is essential for proper functioning of the immune response, protein and DNA synthesis, retinal development, liver function, cell division, gene expression, blood clotting, metalloenzyme function and olfaction (Berg and Shi, 1996; Prasad, 1991).

Zinc plays an important role as a component of a number of metalloenzymes such as carbonic anhydrase, carboxypeptidases and DNA polymerases (Scheideler, 2008). In chickens these enzymes are essential for hens' immune response, skin and wound healing, hormone production and eggshell formation in the hens shell gland (Scheideler, 2008).

2.1.2. Zinc and iron metabolism

Major site of iron regulation are intestine and they are controlling the uptake of dietary iron (Lieu et al., 2001). Dietary iron must be absorbed across the apical membrane, translocated across cytosol and export across the basolateral membrane to be released into the circulation (Lieu et al., 2001). In the intestine iron can exist in two forms: ferrous and ferric iron salts. Conrad et al., (1999) considered ferrous iron salts to be more efficiently absorbed than ferric iron salts which are the main form of dietary inorganic iron.

According to WHO and FAO total absolute recommended requirement for available iron intake for humans ranged between 0.58 mg/day to 3.27 mg/day, but it is necessary to take into consideration that all consumed iron is not absorbed in intestine. Nutritional requirement of iron for broilers is 80 mg/kg in feedd (National Research Council, 1994).

According to NRC, (1998) iron requirement of swine varies from 25 mg/day for suckling pigs to 123 mg/day for the adult swine. Iron requirement of adult cattle and sheep are not based on definitive experiment and little is known about it (Underwood, 1977). Matrone et al., 1957 reported that calves on the zero level of supplemental iron became anemic and gained less weight than those fed either 30 mg or 60 mg of iron per day, and that 30 mg of iron/per day can be considered as minimal nutritional iron requirement for calves. NRC, (2007) recommended 400 mg/day as iron requirement for horses.

Limited ability of body to actively excrete iron has been studied widely by many researchers. Iron is only lost with cells from the skin and the interior surfaces of the body – intestines, urinary tract, and airways (FAO/WHO expert consultation on human vitamin and mineral requirements, 2001). Iron in the excreta is mostly constituted of unabsorbed food iron and in normal human body it ranges between 6 and 16 mg/day (Underwood, 1977). According to Ma et al., (2012) excretion of iron in broiler feces when fed with commercial diet was 294 mg/kg.

Zinc absorption is dependent on concentration and occurs throughout the small intestine (FAO/WHO expert consultation on human vitamin and mineral requirements, 2001). Zinc provided in aqueous solutions to fasting subjects is absorbed efficiently (60–70 percent), but

absorption of zinc from solid diets is less efficient and may vary depending on zinc content and diet composition (Khalid et al., 2014; Turnham, 1990).

There is no specific index for zinc status limits for evaluating zinc requirements (Khalid et al., 2014). According to FAO/WHO expert consultation on human vitamin and mineral requirements (2001) zinc requirements were estimated by using the factorial technique (i.e., by adding the requirements for tissue growth, maintenance, metabolism, and endogenous losses). According to the same source average individual normative requirements for zinc were estimated to be between 59 and 514 (μg/kg body weight/day) for the diet with moderate zinc bio-availability. Broiler daily requirements for Zn were estimated to be 40 mg/kg by conventional method and 32 mg/kg by birds self-selected method (Steinruck and Kirchgessner, 1993).

Mills et al., (1957) found in their experiment that for the growth of suckling calves dietary zinc concentration should be from 8 to 9 mg/kg feed dry matter. Dietary zinc requirement for a 600 kg dairy cow, producing 30 kg milk per day is 40 to 50 mg/kg feed dry matter (Weigand and Kirchgessner, 1982). The zinc nutrient requirement for horses is 400 mg/day according to NRC, (2007). The zinc requirement of mature and sexually reproductive pigs weighing over 50 kg is 50 mg/kg diet, while zinc requirement of growing pigs will vary, depending on their weight, from 60 to 100 mg/kg diet (NRC, 2008).

Losses of zinc from the body include fecal, urinary, loss from desquamated skin cells and sweat and other, small miscellaneous endogenous losses (King and Turnlund, 1989).

In order to meet animal requirements for iron and zinc it is necessary to balance diet adequately using tables with mineral content of feedstuff (Table 1).

Table 1. Iron and zinc content of common feedstuff (dry matter basis) (Kabaija and Little, 1989)

Feedstuff	Fe (mg/kg)	Zinc (mg/kg)
Wheat straw	325	11
Barley straw	1175	12
Linseed straw	103	24
Oats straw	196	17

Maize stover	408	24
Wheat bran	163	75
Sorghum bran	163	25
Sunflower cake	189	94
Rapeseed cake	161	89
Groundnut cake	1183	51
Linseed cake	149	70

2.1.3. Symptoms and prevalence of iron and zinc deficiency

Countries with population depended mainly on cereal-based diet have the greatest chance to develop iron and zinc deficiency, but it can also be widespread among population in industrialized countries. It is estimated that about half of the world's population, despite economic and scientific progress has developed iron and zinc deficiency (King et al., 1989) (Figure 1).

According to World Health Organization (2007) iron deficiency is the most common and widespread nutritional disorder in the world and they estimated that in 2004 iron deficiency anemia resulted in 273 000 deaths: 45% in Southeast Asia, 31% in Africa, 9% in the Eastern Mediterranean, 7% in the Americas, 4%in the Western Pacific, and 3% in Europe, with 97% occurring in low- and middle-income countries. In developing countries of the world, iron deficiency is mostly expressed in the form of iron deficiency anemia while in industrialized countries, for example, prevalence of iron deficiency anemia is much lower and usually varies between 2 to 8 percent, but an absence of iron stores or subnormal serum ferritin values is found in about 20–30 percent of women of fertile age (FAO/WHO expert consultation on human vitamin and mineral requirements, 2001).

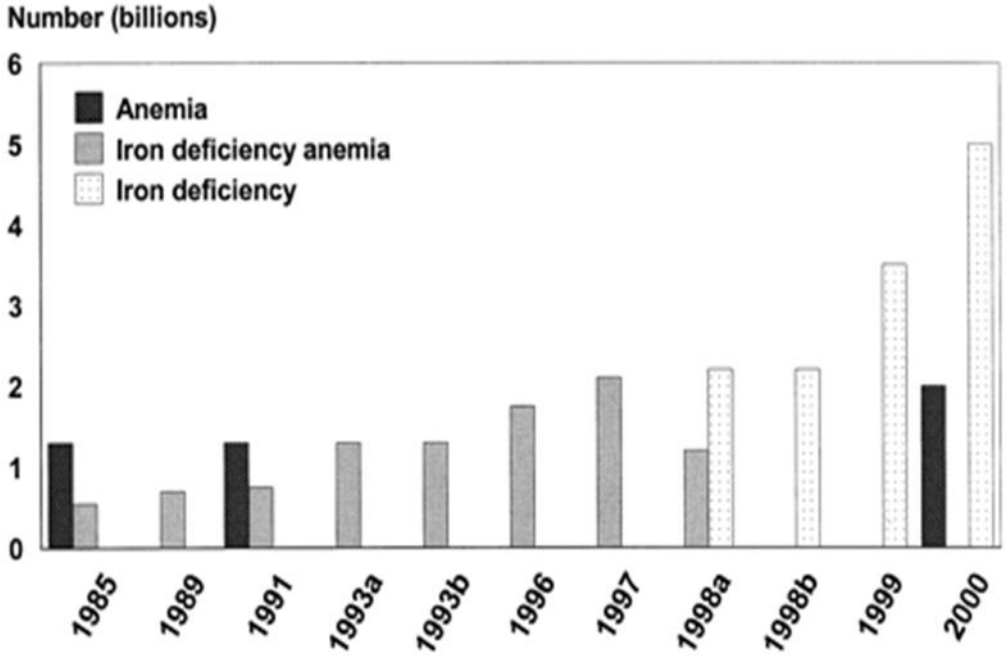

Figure 1. Estimates of the number of people in world with anemia, iron-deficiency anemia, or iron deficiency as stated by various expert groups since 1985 (Source: Stoltzfus, 2001).

Highest prevalence figures for iron deficiency in the world are found in infants, children, teenagers, and women of childbearing age (FAO/WHO expert consultation on human vitamin and mineral requirements, 2001).

Symptoms of iron deficiency in human adults include listlessness and fatigue, palpitation on exertion, and sometimes a sore tongue, angular stomatitis, dysphagia, and koilonychia (Underwood, 1977). In children anorexia, depressed growth, and decreased resistance to infection are commonly observed, the same as in other young, growing, iron-deficient animals, but oral lesions and nail changes that can be noticed on animals are rare (Underwood, 1977). Iron deficiency negatively influences the normal defense systems against infections and as a result of a reduced formation of T lymphocytes cell-mediated immunologic response is impaired (FAO/WHO expert consultation on human vitamin and mineral requirements, 2001).

Iron deficiency occurs in baby pigs when they have no access to other sources of iron rather than sow's milk and in older pigs when fed with high copper levels feed (Underwood, 1977).

Literature reviews estimates that 20% of the world’s population may be at risk of inadequate dietary intake of zinc (Figure 2) and the populations at highest risk are located in South and

Southeast Asia, Sub-Saharan Africa, Central America, and the Andean region (Khalid et al., 2014).

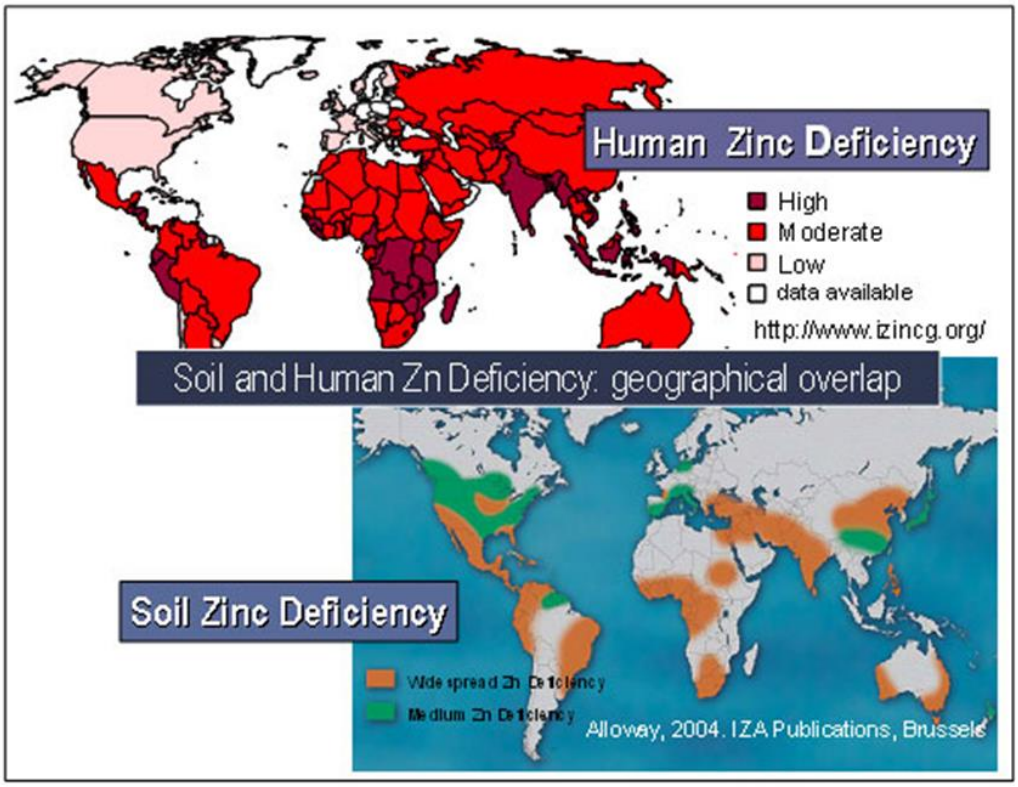

Figure 2. Close geographical linkage between soil zinc deficiency and human zinc deficiency (Source: Alloway, 2004; Cakmak, 2009; www.izincg.org)

Zinc deficiency can be divided in two types:

1. Primary deficiency (result from inadequate dietary intake of zinc (Khalid et al., 2014);

2. Secondary deficiency develops in some people with cirrhosis, mal-absorption syndromes, sickle cell anemia, conditions of increased zinc loss, such as severe burns or major surgery, chronic diarrhea or diabetes, HIV and AIDS, and during prolonged parenteral nutrition (Khalid et al., 2014; Prasad, 1999)

The symptoms of severe zinc deficiency in humans include growth retardation, delayed sexual and bone maturation, skin lesions, diarrhea, alopecia, impaired appetite, increased susceptibility to infections mediated via defects in the immune system, and the appearance of behavioral changes (Hambidge, 1987; FAO/WHO expert consultation on human vitamin and mineral requirements, 2001).

Manifestation of zinc deficiency in animals include loss of hair, thickening and hyperkeratinization of the epidermis, testicular atrophy (Prasad, 1991), skin parakeratosis, reduced growth, general debility, lethargy, and increased susceptibility to infection (Miller, 1970).

2.1.4. Phytate as a main inhibitor of iron and zinc absorption

"The bioavailability of a nutrient can be defined as the proportion of the total nutrient content in a food, meal or diet that is used for normal metabolic functions" (Lestienne et al., 2005). Bioavailability of iron and zinc in cereals is often reduced because of the presence of anti-nutritional factors. Anti-nutritional factors can cause inefficient and variable absorption from the diet, for instance iron (<1–30%) and zinc (<15–50%) (Lestienne et al., 2005). The main inhibitor of iron and zinc absorption is myoinositol hexaphosphate (phytate)(Figure 3).

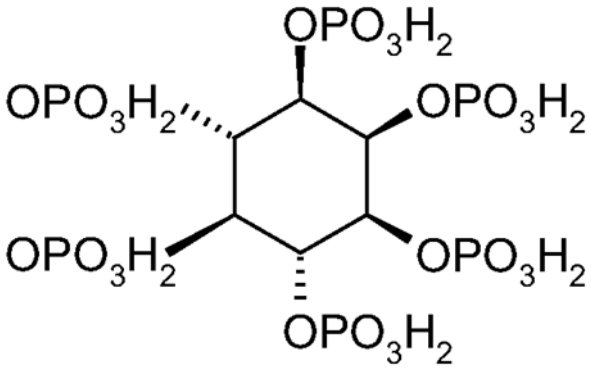

Figure 3. Myoinositol hexaphosphate (Source: Grases, 2008)

The presence of phytate in food and feed has been connected with reduced mineral absorption due to the structure of phytate which has high density of negatively charged phosphate groups which form very stable complexes with mineral ions causing non-availability for intestinal absorption (Walter et al., 2002).

The effect of phytic acid on zinc availability has been studied for over 50 years. O'Deli and Savage, (1960) were first one to suggest that naturally occurring phytic acid in plant protein might reduce the availability of dietary zinc. Later on Lonnerdal et al., (1988) found that, zinc absorption was significantly improved when the phytate was removed from soy protein isolate by a precipitation process.

Brown et al., (2001) showed that if zinc content of polutry meat is 1.8 – 3.0 mg/100 g and phytate content is 0, then absorbable zinc mg/100 g would range between 0.9 – 1.5 mg/100 g. On the other hand if zinc content in wheat is between 0.5 – 3.2 mg/100 g and phytate content is 211 – 618 mg/100 g then absorbable zinc would be between 0.1 – 0,. mg/ 100 g.

In the study by Hurell et al., (2003) it was concluded that when the foods were reconstituted with water, dephytinization increased iron absorption from wheat from 0.99% to 11.54% ($P < 0.0001$) (Table 2)

Table 2. Influence of phytic acid degradation on iron absorption from wheat porridges (Source: Hurell et al., 2003).

Cereal	Meals	Iron absorption[2] (% of dose)	Absorption ratio versus native phytate[23]
Wheat	Wheat, native phytate	0.99	-
Wheat	Wheat – milk, native phytate	1.30	-
Wheat	Wheat - dephytinized	11.54	11.605[4]
Wheat	Wheat – milk, dephytinized	1.63	1.26

[1]Geometric $\bar{x}$; range in parentheses.

[2]Geometric $\bar{x}$; −1 SE and +1 SE in parentheses.

[3]Absorption ratio calculated as iron absorption from meal with no phytic acid divided by iron absorption from the same meal containing its native phytic acid.

[4]$P < 0.0001$.

2.1.5. Strategies and solutions to combat iron and zinc deficiencies

Because iron and zinc deficiency is common for both, developed and developing countries, it is necessary to develop methods to combat these deficiencies. The two major nutrition related strategies are:

1. Fortification and biofortification
2. Dephytinization

WHO/FAO defined fortification as "the practice of deliberately increasing the content of an essential micronutrient, for example vitamins and minerals (including trace elements) in a food irrespective of whether the nutrients were originally in the food before processing or not, so as to improve the nutritional quality of the food supply and to provide a public health benefit with minimal risk to health".

Micronutrient fortification of foods and condiments is an inexpensive and highly cost effective strategy for improvement and protection of the health and nutritional status of the populations (Khalid et al., 2014).

Iron fortification has two technical barriers to overcome. The first barrier is selection of an iron compound that causes no sensory changes but it is properly absorbed and the second one is to overcome the inhibitory effect of phytic acid and other food components on iron absorption in the intestines (Hurrell, 2002).

Biofortification can be defined as development of micronutrient-dense staple crops using the best traditional breeding practices and modern biotechnology (Nestel et al., 2006). The advantage of iron and zinc biofortification over regular fortification is the fact that biofortification is oriented towards enhancing minerals in the plant while it is growing. In this way biofortified food will be available for broad range of people, especially poor people who do not have access to commercially fortified food.

According to Cakmak, (2007) biofortification has multiple advantages which include: regular daily intake of a consistent and large amount of food staples by all family members, low recurrent costs after the initial cost of development of fortified seeds, high sustainability of biofortified crop systems and providing a feasible means of reaching undernourished populations in relatively remote rural areas.

Cakmak, (2007) has shown that foliar or combined soil + foliar application of zinc fertilizers under field conditions are highly effective and very practical way to maximize uptake and accumulation of zinc in whole wheat grain, raising concentration up to 60 mg Zn kg-1. Applying zinc fertilizers to wheat grown in field in Central Anatolia improved not only productivity, but also grain zinc concentration (Yilmaz et al. 1997).

Dephytinization can be described as a process of adding an exogenous phytase or by activating the naturally occurring plant phytases in order to increase mineral availability and reduce mineral deficiency (Hurell, 2004; Frontela et al., 2009). Frontela et al. (2009) found

that dephytinization of infant cereals significantly increased ($P < 0.05$) the cell uptake efficiency (from 0.66%-6.05% to 3.93%-13%), retention (from 6.04% - 16.68% to 14.75% - 20.14%) and transport efficiency (from 0.14%-2.21% to 1.47%-6.02%), of iron, and the uptake efficiency (from 5.0%-35.4% to 7.3%-41.6%) and retention (from 4.05%-20.53% to 14.45%-61.3%) of zinc.

Benefits of phytase addition to animal feed were studied extensively by many researchers. Sebastian et al., (1996) found that phytase supplementation increased body weight in male and female chickens by 13.2 and 5.8 %, respectively, and relative retention of total Zn by 62.3 percentage units. When microbial phytase was added to low – P diet, absorbability of P increased by 24 % and availability of P increased to over 60 % (Simons et al., 1990).

2.2. Chicken meat and its importance as a source of iron and zinc

"The world chicken industry is a growing part of global agribusiness and also one of the most dynamic parts of world agribusiness trade" (Aho, 2002). According to FAO Statistical Yearbook 2012, chicken meat production increased from 58,5 million tonnes in 2000 to 91,6 million tonnes in 2012, and it is predicted that it will reach 94,8 million tonnes in 2014.

Table 3. Chicken meat production (million tonnes)

Region	2000	2005	2007	2008	2009	2010	2011	2012E	2013F	2014F
Africa	2.8	3.3	3.7	4.0	4.2	4.5	4.6	4.7	4.7	4.8
Americas	27.1	32.7	35.0	37.4	36.7	38.6	39.9	40.4	41.2	41.9
Asia	18.6	22.4	25.0	26.2	28.0	29.1	29.8	30.3	30.7	31.2
Europe	9.3	10.9	11.6	12.1	13.3	13.9	14.6	14.9	15.2	15.5
Oceania	0.7	0.9	1.0	1.0	1.0	1.1	1.2	1.3	1.3	1.4
WORLD	**58.5**	**70.2**	**76.2**	**80.6**	**83.2**	**87.2**	**90.0**	**91.6**	**93.2**	**94.8**

E-estimates; F-forecast

Source: FAO Statistical Yearbook, 2012; www.thepoultrysite.com;

Table 3. Shows that chicken meat production is increasing rapidly in Africa and Asia where many countries are still developing. Demand for meat products is rising faster than that for

cereals and other supplies as per capita income rises in developing countries (Bender, 2002). In FAO Poultry developing review (2013) it was concluded that livestock is fundamental to the livelihoods of about one billion of the world's poorest people and that rural poultry, in particular, is essential for the livelihood of many resource-poor farmers often being the only asset they possess. The same review mentioned that rural poultry makes up about 80 percent of poultry stocks in low-income food-deficit countries and significantly contributes to: improving human nutrition, providing food (eggs and meat) with high quality nutrients and micronutrients and generating a small income and savings, especially for women, thus enhancing the capacity to cope with shocks and reducing economic vulnerability.

In the countries were consumption of meat per inhabitant is below 10 kg, many people can be considered malnourished. To effectively combat such malnutrition WHO and FAO recommended 20 g of animal protein per person per day or 7.3 kg per year. FAO predicted that this can be achieved by an annual consumption of 33 kg lean meat or 45 kg fish or 60 kg eggs or 230 kg milk.

Chicken meat production is the best solution for increasing meat consumption in any country because it is cheapest meat to produce.

The reason way it would be more preferable to increase meat consumption, rather than cereals consumption is because meat provides highly available iron and zinc. Meat protein may enhance zinc bioavailability because zinc absorption was increased by additional protein in a meal (Hunt et al., 1995). This effect can be depended on phosphorus levels in meat. Greger and Snedeker (1980) found that the level of dietary protein and phosphorus all statistically affected fecal zinc excretion and that loss of zinc in the feces is lowest in the subjects when fed the high protein and moderate phosphorus diet.

Substantial research with single meals suggests excellent iron absorption from meat, both because of highly bioavailable iron in the heme form, as well as unidentified factors in meat that promote heme and non-heme meat absorption (Hunt et al., 1995).

2.3. Review on the effect of content of iron and zinc in feed on their concentration in animal body

In recent years, studies have shown that increased levels of iron and zinc in feed have effect on increase of these minerals in body tissues until certain levels of supplementation are provided. In practice, producers usually formulate diets in order to exceed mineral requirements of animal, in the case of higher dietary mineral need. Study of Ma et al., (2012) showed that increase in iron levels from dietary recommended 80 mg/kg diet to 160 mg/kg diet in 21 day old broiler chicken did not resulted in increased body weight and did not have effect on increase of zinc concentration in serum, liver, breast muscle and tibia. However, increase in iron levels in the diet did induced increase in iron levels in chicken serum, liver, breast muscle and tibia bone and it did enhanced iron concentration in feces. The results of Ma et al. (2012) are in accordance with results of Chreech et al., (2004) who also observed that increased dietary iron level (150 mg/kg diet) resulted in a linear increase of fecal iron excretion in piglets. In the study of Cao et al., (1996) it was found that when 400, 600, or 800 mg/kg Fe is added as reagent grade FeSO4.7H2O to broiler chickens diet, feed intake and body weight gain are depressed, but kidney, liver and bone concentration increased linearly with increase in dietary iron.

When it comes to the increase in tissue zinc concentration as the consequences of increased zinc levels in the diet, research of Bao et al., (2009) found that when dietary levels of zinc were increased from 0 to 40 mg/kg diet, body weight gain and total tibia iron and zinc contents increased linearly, but zinc supplementation had no effect on bone width and strength. This is confirmed in the study of Mohanna and Nys, (1999) were tibia and plasma zinc concentration also increased linearly when zinc dietary content was increased from 20 to 190 mg/kg diet. In this study it was also confirmed that beneficial effect of higher dietary zinc content reached its maximum at 75 mg/kg diet and that further increase in zinc content caused decrease in zinc retention which was 8% for the diet that contained 190 mg Zn/kg diet.

3. Materials and methods

3.1. Field experiment

The main component of broiler diets was mineral improved wheat produced in the field experiment performed in 2012 in Croatia. In this experiment two winter wheat varieties were used and two types of treatment were applied. First variety is Srpanjka. Srpanjka is Croatian most popular wheat variety, with genetic yield potential greater than 10 Mg/ha and high tolerance toward widespread wheat diseases. Second variety is Serbian variety Simonida with average genetic yield potential of 8 Mg/ha. Varieties were seeded in 20 m2 plot size on calcerous soil in Pannonian basin and treated with two different mineral supplements (Zn and Se). Zn was applied in the form of Zn sulfate – 1.5 kg Zn ha-1 (6.6 kg ZnSO4×7H2O ha-1). Zn treatment was foliar application with 600 L ha-1 (0.25 % Zn w/v) + 0.1 % surfactant HERBOVIT v/v application at half of heading stage (Feekes 10.3) to beginning of flowering Feekes (10.51). Selenium treatment was soil application in the form of selenate, but because results regarding selenium addition to feed will not be discussed in this paper, further explanation will not be available. The plots were harvested with specialized combine harvester and grain was packed and forwarded to Norway.

3.2. Diet formulation and feed production

The experimental design consisted of 9 diets. The diets were as follows:

1. Commercial starter diet was used from day 1 to – day 7 posthatch and it was given to all broiler chickens in the form of pellets. Diet was purchased in the shop;
2. Starter micro mineral deficient diet was used from day 7 to – day 11 posthatch and it was given to broiler chickens as a mash feed;

The rest of the diets were supplied to broiler chickens from day 11 to – day 22 posthatch in a mesh state.

3. Commercial grower diet (control diet) was purchased in the shop and it contained 190 mg Fe/kg diet and 93 mg Zn/kg diet (Table 4).
4. Grower diet with wheat Srpanjka ($ZnSO_4$ treatment), supplemented with phytase, contained 210 mg Fe/kg diet and 46 mg Zn/kg diet (Table 4).
5. Grower diet with wheat Simonida ($ZnSO_4$ treatment), supplemented with phytase, contained 240 mg Fe/kg diet and 52 mg Zn/kg diet (Table 4).

6. Grower diet with wheat Srpanjka and Simonida (Se_2 soil treatment), supplemented with phytase, contained 140 mg Fe/kg diet and 37 mg Zn/kg diet (Table 4).
7. Grower diet with wheat Srpanjka (ZnSO4 treatment), without phytase, contained 190 mg Fe/kg diet and 49 mg Zn/kg diet (Table 4).
8. Grower diet with wheat Simonida (ZnSO4 treatment), without phytase, contained 220 mg Fe/kg diet and 57 mg Zn/kg diet (Table 4).
9. Grower diet with wheat Srpanjka and Simonida (Se2 soil treatment), without phytase, contained 170 mg Fe/kg diet and 38 mg Zn/kg diet (Table 4).

Table 4. Levels of iron and zinc in different diets (mg/kg) assigned to broiler chickens from day 11 to – day 22 posthatch.

Diets	Fe (mg/kg)	Zn (mg/kg)
Control	190	93
Srpanjka (ZnSO4), with phytase	210	46
Simonida (ZnSO4), with phytase	240	52
Srpanjka and Simonida (Se2 soil), with phytase	140	37
Srpanjka (ZnSO4), no phytase	190	49
Simonida (ZnSO4), no phytase	220	57
Srpanjka and Simonida (Se2 soil), no phytase	170	38

All diets except commercial starter and grower diet were formulated and produced at Fôrtek (Center for Feed Technology) at Norwegian University of Life sciences in Ås. Diets were formulated using Ross Broiler management Manual (2009) to meet nutritional requirements of broiler chickens.

Table 5. Composition of starter micro mineral deficient diet.

Raw material	Proportion (%)	Amount (kg/ treatment)
Norwegian wheat grain	58.59	9
SBM[1]	32.69	5.02
Soybean oil	3.96	0.6
Limestone	1.91	0.293
MCP[2]	1.05	0.161
DL-methionine	0.2	0.03
L-lysine	0.2	0.03
L-threonine	0.1	0.015
Salt	0.25	0.038
Sodium bicarbonate	0.17	0.025
Choline chloride	0.12	0.018
Vitamin A	0.07	0.01
Vitamin D3	0.07	0.01
Vitamin E	0.04	0.006
ADKB	0.08	0.012
Titanium dioxide	0.5	0.076
Total	100	15

[1]SBM denotes soy bean meal

[2] MCP mono calcium phosphate

Starter micro mineral deficient diet was formulated in order to achieve following composition: 22 % crude protein, 12.60 MJ/kg metabolic energy, 1 % Ca, 0.5 % P, 0.16 % Na, 1.44 % Lysine, 0.51 % Methionine, 0.25 % Tryptophan and 0.93 % Threonine (Table 5).

Enzyme phytase used in this experiment was Phyzyme XP 5000G (solid state).

Titanium dioxide was used as indigestible marker and added to diets containing wheat from field experiment.

Commercial starter diet and commercial grower diet (control diet) were purchased at Felleskøpet.

Table 6. Composition of grower diets in amount (kg/treatment)

Raw material	Diet [1]	Diet [2]	Diet [3]	Diet [4]	Diet [5]	Diet [6]
Wheat	11	11	21.5	11	11	21.5
SBM[7]	6.795	6.795	13.28	6.774	6.774	13.24
Soybean Oil	1.391	1.391	2.71	1.381	1.381	2.70
Limestone	0.347	0.347	0.679	0.346	0.346	0.667
MCP[8]	0.180	0.180	0.353	0.180	0.180	0.352
DL-methionine	0.041	0.040	0.08	0.041	0.041	0.081
L- lysine	0.040	0.040	0.078	0.040	0.040	0.078
L-threonine	0.02	0.02	0.039	0.02	0.02	0.039
Salt	0.05	0.05	0.098	0.05	0.05	0.097
Sodium bicarbonate	0.034	0.034	0.068	0.034	0.034	0.068
Choline chloride	0.024	0.024	0.047	0.024	0.024	0.047
Vitamin A	0.014	0.014	0.027	0.014	0.014	0.027
Vitamin D3	0.014	0.014	0.027	0.014	0.014	0.027
Vitamin E	0.008	0.008	0.015	0.008	0.008	0.015
ADKB	0.016	0.016	0.031	0.016	0.016	0.031
Titanium oxide	0.100	0.100	0.196	0.100	0.100	0.195
Enzyme-phytase	0.004	0.007	0.007	-	-	-
Total	20	20	39	20	20	39

[1]Srpanjka (ZnSO4), with phytase

[2]Simonida (ZnSO4), with phytase

[3]Srpanjka and Simonida (Se2 soil), with phytase

[4]Srpanjka (ZnSO4), no phytase

[5]Simonida (ZnSO4), no phytase

[6]Srpanjka and Simonida (Se2 soil), no phytase

[7]SBM denotes soy bean meal

[8] MCP mono calcium phosphate

Grower diets (Table 6) were balanced in order to achieve following composition: 22 % crude protein, 13.3 MJ/kg metabolic energy, 0.9 % Ca, 0.45 % P, 0.16 % Na, 1.25 % Lysine, 0.45 % Methionine, 0.22 % Tryptophan and 0.82 % Threonine.

Wheat and soybean meal were ground on small scale grinding mill (Model: KM1, 4 kW/220V, Skiold A/S Kjeldgaardsvej 3 Saeby Denmark, with grinding capacity 20-30 kg/5 minutes and sieve hole size 3-mm) and measured on scale in order to separate necessary amount predicted in diet formulation. Commercial grower diet was grinded in order to avoid difference in feed appearance. After measurement all wheat and soybean meal were divided, depending on the diet they composed, among 7 plastic boxes. Micro elements were also weighed manually on scale and added to the boxes with wheat and soybean meal. Ingredients were mixed together for two minutes to form balanced diet in self-made mixer at FôrTek (Idecon, 40 l, twin shaft paddle) (Figure 4). After initial mixing, ingredients were mixed for second time, but now with the addition of soybean oil during the mixing. For the purpose of oil spraying, self-made tank from FôrTek was used. The tank works on the principle of pressure release when it sprays oil on the feed and it is necessary to pump air into the tank (to achieve 4 bars) and of course to pour oil first. Nozzle used for this process was 6503 (angel of spraying 65, size 03, spraying capacity for oil 1.1 l/min) Spraying System Co, Wheaton, Illinois, USA. Ingredients were mixed for two-three more minutes and after mixing they were packed into plastic bags. All diets were given to the chickens in a mesh state. Representative samples of each diet were taken.

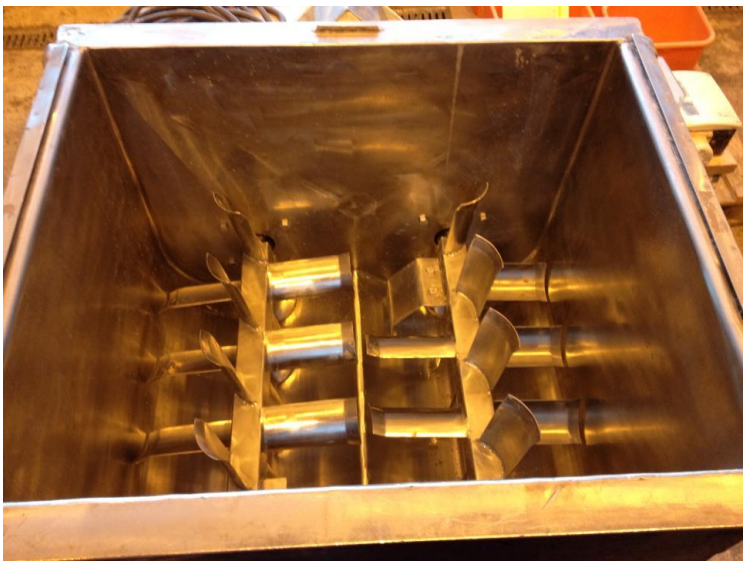

Figure 4. Idecon, 40 l, twin shaft paddle, self made mixer.

3.3. Feeding experiment

Experiment started on November 14, 2013 when ninety day-old male broiler chickens (Ross 308) arrived to Chicken house (Animal production experimental centers at Norwegian University of Life Sciences). Chickens were weighed for the first time and placed in heated, thermostatically controlled brooder cage, maintained on 24-h constant-fluorescent light schedule and fed with a commercial starter diet until 7 day of life. At 7 day of age diet was changed to starter micro mineral deficient diet which was retained for 4 days. At 11 days of age, the chickens were weighed for the second time and 70 of them were transferred to individual mesh floor cages and assigned to one of the 7 final grower diets. Each of the six experimental grower diets and one commercial grower diet were inserted in paper feed boxes, weighed and placed in front of the 10 cages (Figure 5). On average there was 1.2 kg of feed in every box. Chickens fed with the same diet were dispersed all across room.

Figure 5. Chicken arrangement

At 19 day of age all chickens were weighed for the third time and number was reduced to 49 (7 replicates per diet). The rest of the chickens were removed from their cages. The weight of the feed boxes was also recorded and trays for the excreta collection were placed under every cage. From 19 to 22 day of age, total excreta from each cage was collected daily and frozen at

-23 °C. Termination of feeding experiment was performed on 22 day of age when all chickens and feed boxes were weighed for the last time and feed boxes were removed from the cages.

Chickens were killed by dislocation of their neck and dissected immediately. Samples of liver and left breast muscle were individually collected into plastic bags and frozen at -23 °C.

Through all feeding experiment chickens had ad libitum access to feed and deionized water. Room temperature was maintained at 33 °C for the first 7 days of age, at 29 °C from 7 to 11 day of age and at 26 °C for the last 11 days of the experiment.

3.4. Sample analyses

3.4.1. Dry matter analysis

Analysis of dry matter was performed on feed, excreta, liver and breast muscle samples. Excreta samples were stored in buckets and frozen. Before dry matter analysis, weight of each bucket was measured and content of buckets was defrosted and homogenized by simple mixer. About 15 g of representative sample was taken and placed in the cruciables, weighed and then dried in the oven for 24 h at 104 °C. After drying, samples were cooled down in desiccator and measurement of net weight of the dry samples was performed. This procedure was similar for all samples except initial handling and weight of samples. Before sampling of liver and breast muscle, their total weight was measured and 1 g of representative sample was cut down with the scalpel and placed in the cruciables. As regard to the feed, 2 g of representative samples were taken for dry matter analysis.

After drying, excreta samples were grinded by mortar and pestle and packed into small glass bottles. This was done for the purpose of further analysis.

3.4.2. Sample digestion procedure

Samples of excreta, feed, liver and breast muscle were digested at the Department of Environmental Sciences – Norwegian University of Life Sciences by Ultra Clave (MLS 1200 mega microwave digestion unit, Milstone, Sorisole, Italy). For the optimal digestion it was necessary to prepare samples carefully and to avoid any contamination. Preparation was not the same for all samples but they were all placed into Teflon tetrafluorethylene vessel and weighed on an analytical scale.

Excreta samples (dry state): 0.25 g sample + 2 ml deionized water + 5 ml HNO3 + 1 ml HF

Feed samples (fresh state): 0.25 g sample + 2 ml deionized water + 5 ml HNO_3 + 1 ml HF

Liver and breast muscle samples (fresh state): 1 g sample + 5 ml HNO_3

Standard reference material was used for digestion and prepared in the same matrix as samples:

- 1577b (Bovine liver)
- GBW07603 (Bush branches and leaves)
- DORM-3 (Dogfish muscle)
- 8415 (Whole egg powder)
- 1567a (Wheat flour)
- 1515 (Apple leaves)

Digestion of samples was performed in Ultra Clave, and the the run time was 2 hours. After full digestion, samples were filtered into a 50 mL flask and diluted to 50 mL with deionized water. Samples digested with HF acid were rediluted (1 mL of diluted sample was filtered into 15 mL flask and diluted to 10 mL with deionized water).

All solutions (excreta, feed, liver and breast muscle digest) were analyzed for Fe, Zn and Ti content against standard reference material. The elements were determined by Agilent 8800 ICP-MS. Ti is quantified as a mass shift reaction with oxygen. Q2 = Q1+16, Q1 Ti = 47, Fe and Zn are quantified in He-KED at mass 56 and 66.

3.4.3 Marker method for measurement of the digestibility of iron and zinc

Digestibility of iron and zinc were calculated as follow:

$$\text{Digestibility} = 1 - \left(\frac{\%\ \text{marker in feed} * \%\ \text{nutrient in excreta}}{\%\ \text{marker in excreta} * \%\ \text{nutrient in feed}}\right)$$

3.5. Statistical analysis

Statistical analyses were performed using R (version 3.0.3). The data were analyzed using 1-way ANOVA with diet as the factor. The significance of difference between means was determined using the Tukey HSD post hoc pair wise test and difference was considered significant at ($P < 0.05$).

4. Results

4.1. Growth performance

Despite the fact that 70 broiler chickens were randomly picked up and assigned to the one of seven diet on day 11, chickens assigned to diet with wheat Simonida(ZnSO4) - with phytase had significantly lower weight gain when compared to chickens assigned to control diet and diet with wheat Srpanjka and Simonida (Se2 soil) - no phytase ($P < 0.05$)(Table 7).

In the period between day 11 and day 22 it is observed that feed intake and weight gain were significantly increased in the chickens fed with experimental diets when compared to chickens fed with control diet. The statistical difference between control diet and experimental diets (wheat Srpanjka (ZnSO4) - with phytase, wheat Srpanjka and Simonida (Se2 soil) - with phytase, wheat Srpanjka (ZnSO4) - no phytase, wheat Simonida (ZnSO4) - no phytase, wheat Srpanjka and Simonida (Se2 soil) - no phytase) was significant ($P < 0.001$) and the same was true between control diet and experimental diet with wheat Simonida (ZnSO4) - with phytase ($P < 0.005$) (Table 7).

Feed conversion rate (FCR) was only significantly different ($P < 0.001$) between control diet and all experimental diets, and there was no significant effect of different experimental diets on FCR ($P > 0.05$) (Table 7).

Table 7. Effect of different diets on feed intake, weight gain and FCR of broilers (0 to 22 day)

	11 d	11-21 d[1]		
Diet	Weight (g/bird)	Feed intake (g/bird)	Weight gain (g/ bird)	FCR[2]
Diet [3]	276[b]	627[a]	383[a]	1.638[a]
Diet [4]	272[ab]	821[b]	642[b]	1.278[b]
Diet [5]	246[a]	791[b]	618[b]	1.279[b]
Diet [6]	266[ab]	863[b]	688[b]	1.253[b]
Diet [7]	263[ab]	834[b]	657[b]	1.269[b]
Diet [8]	272[ab]	863[b]	679[b]	1.271[b]
Diet [9]	276[b]	861[b]	672[b]	1.280[b]
P-value	0.031	<0.001	<0.001	<0.001

[1] Data represent means of 7 replicate groups of 7 broiler chickens

[a-b] Means sharing the same superscript are not significantly different from each other (Tukey's HSD, $P < 0.05$)

[2]FCR – feed conversion ration

[3]Control diet

[4]Wheat Srpanjka (ZnSO4) - with phytase

[5]Wheat Simonida (ZnSO4) - with phytase

[6]Wheat Srpanjka and Simonida (Se2 soil) - with phytase

[7]Wheat Srpanjka (ZnSO4) - no phytase

[8]Wheat Simonida (ZnSO4) - no phytase

[9]Wheat Srpanjka and Simonida (Se2 soil) - no phytase

4.2. Mineral concentration in breast muscle

Table 8. shows that although iron and zinc concentration in breast muscle were higher in chickens fed with experimental diets compared to chickens fed with control diet, statistical analyses demonstrated no significant difference ($P > 0.05$) between them.

Table 8. Effect of different diets on mineral concentration in breast muscle at 22 day of age [1](mg/kg of dry breast muscle)

Diet	Fe (mg/kg)	Zn (mg/kg)
Control diet	3.52	6.00
Wheat Srpanjka (ZnSO4) - with phytase	4.49	6.68
Wheat Simonida (ZnSO4) - with phytase	3.76	6.64
Wheat Srpanjka and Simonida (Se2 soil) - with phytase	4.58	7.12
Wheat Srpanjka (ZnSO4) - no phytase	3.92	6.37
Wheat Simonida (ZnSO4) - no phytase	4.28	6.81
Wheat Srpanjka and Simonida (Se2 soil) - no phytase	3.91	6.64
P-value	0.222	0.187

[1] Data represent means of 7 replicate groups of 7 broiler chickens

4.3. Mineral concentration in liver

Average concentration of iron in the chicken liver ranged between 162.06 and 253.25 mg/kg of dry liver (Table 9), but there was no significant difference ($P > 0.05$) between iron concentration in the liver. Table also shows that there is no significant difference ($P > 0.05$) between zinc levels when chickens are fed with different diets.

Table 9. Effect of different diets on mineral concentration in liver at 21 day of age[1] (mg/kg of dry liver)

Diet	Fe (mg/kg)	Zn (mg/kg)
Control diet	162.06	28.68
Wheat Srpanjka (ZnSO4) - with phytase	207.26	25.25
Simonida (ZnSO4), with phytase	185.90	25.93

Srpanjka and Simonida (Se2 soil), with phytase	253.25	24.39
Srpanjka (ZnSO4), no phytase	232.25	25.81
Simonida (ZnSO4), no phytase	175.91	25.59
Srpanjka and Simonida (Se2 soil), no phytase	203.38	25.19
P-value	0.409	0.156

[1] Data represent means of 7 replicate groups of 7 broiler chickens

4.4 Fecal mineral excretion

Analyzed values of iron and zinc for fecal samples are presented in (Table 10). Fecal iron concentration was significantly different ($P < 0.01$) between control diet and diet with wheat Srpanjka and Simonida (Se2 soil) - with phytase. The difference was not significant among other diets.

(Table 10) also shows that there is significant difference ($P < 0.001$) between fecal zinc concentration in control diet and all experimental diets. The difference was also presented between diet with wheat Srpanjka and Simonida (Se2 soil) - with phytase and diet with wheat Srpanjka (ZnSO4) - with phytase ($P < 0.05$), diet with wheat Srpanjka and Simonida (Se2 soil) - with phytase and diet with wheat Simonida (ZnSO4) - with phytase ($P < 0.001$), diet with wheat Srpanjka and Simonida (Se2 soil) - with phytase and diet with what Simonida (ZnSO4), no phytase ($P < 0.001$), diet with wheat Simonida (ZnSO4) - with phytase and diet with wheat Srpanjka (ZnSO4) - no phytase ($P < 0.05$), diet with wheat Simonida (ZnSO4) - with phytase and diet with heat Srpanjka and Simonida (Se2 soil) - no phytase ($P < 0.001$) and between diet with wheat Simonida (ZnSO4), no phytase and diet with wheat Srpanjka and Simonida (Se2 soil) - no phytase ($P < 0.001$).

Table 10. Effect of different diets on fecal mineral excretion [1](g/kg of dry feces from day 19 – 22)

Diet	Fe (mg/kg)	Zn (mg/kg)
Control diet	538[a]	397[a]

Wheat Srpanjka ($ZnSO_4$) - with phytase	477^{ab}	155^{ce}
Wheat Simonida ($ZnSO_4$) - with phytase	500^{ab}	192^{c}
Wheat Srpanjka and Simonida (Se_2 soil) - with phytase	387^{b}	108^{b}
Wheat Srpanjka ($ZnSO_4$) - no phytase	444^{ab}	147^{bde}
Wheat Simonida ($ZnSO_4$) - no phytase	471^{ab}	182^{cd}
Wheat Srpanjka and Simonida (Se_2 soil) - no phytase	440^{ab}	114^{be}
P-value	0.016	< 0.001

[a-e] Means sharing the same superscript are not significantly different from each other (Tukey's HSD, P < 0.05)

[1] Data represent means of 7 replicate groups of 7 broiler chickens

4.5. Digestibility of iron and zinc

Table 11. Apparent digestibility of iron and zinc using external marker[1].

Diet	Fe	Zn
Wheat Srpanjka (ZnSO4) - with phytase	0.390^{ac}	0.042^{ab}
Wheat Simonida (ZnSO4) - with phytase	0.405^{a}	$- 0.085^{a}$
Wheat Srpanjka and Simonida (Se2 soil) - with phytase	0.207^{bc}	0.410^{b}
Wheat Srpanjka (ZnSO4) - no phytase	0.364^{ac}	0.234^{ab}
Wheat Simonida (ZnSO4) - no phytase	0.448^{a}	0.248^{ab}
Wheat Srpanjka and Simonida (Se2 soil) - no phytase	0.256^{bc}	0.300^{ab}
P-value	< 0.001	< 0.05

[a-b] Means sharing the same superscript are not significantly different from each other (Tukey's HSD, P < 0.05)

[1] Data represent means of 7 replicate groups of 7 broiler chickens

Table 11. shows the effect of different diets on digestibility of iron and zinc in broilers. Compared with diet with wheat Srpanjka and Simonida (Se2 soil) - with phytase whose average digestibility was 0.2, diet with wheat Srpanjka (ZnSO4) - with phytase ($P < 0.01$), diet with wheat Simonida (ZnSO4) - with phytase ($P < 0.01$), diet with wheat Srpanjka (ZnSO4) - no phytase ($P < 0.05$) and diet with wheat Simonida (ZnSO4) - no phytase ($P < 0.001$) had significantly higher digestibility. As regards to the diet with wheat Srpanjka and Simonida (Se2 soil) - no phytase, with an average digestibility 0.25, significant difference was noticed when it was compared to diet with wheat Simonida (ZnSO4) - with phytase ($P < 0.05$) and diet with wheat Simonida (ZnSO4) - no phytase ($P < 0.01$).

Significant difference in bioavailability of zinc was presented only between diet with wheat Simonida (ZnSO4) - with phytase and diet with wheat Srpanjka and Simonida (Se2 soil) - with phytase ($P < 0.05$).

Control diet had no titanium oxide as external marker in the formulation because it was not produced in Fortek, but bought in the commercial shop.

4.6. Analyses of fungal phytase (XP 5000) addition effect

Table 12. Analysis across studies on the extent to which microbial phytase supplementation in 3 diets for broiler chickens influenced weight gain, feed intake, feed conversion ratio, mineral retention in breast muscle and liver, fecal mineral excretion and digestibility of minerals in comparison to unsupplemented diets;

	Diet		
	Phytase	No phytase	P value
Weight gain (g)	916	947	0.422
Feed intake (g)	825	852	0.464
FCR	1.272	1.277	0.803
Fe retention in breast muscle (mg/kg)	4.281	4.16	0.585
Zn retention in breast muscle	6.81	6.61	0.396
Fe retention in liver (g/kg)	0.21	0.20	0.670

Zn retention in liver (g/kg)	25.19	25.53	0.663
Fe fecal excretion (g/kg)	0.45	0.46	0.761
Zn fecal excretion (g/kg)	0.15	0.14	0.728
Fe digestibility	0.33	0.35	0.821
Zn digestibility	0.12	0.26	0.148

There was no effect ($P > 0.05$) of phytase addition on weight gain, feed intake, feed conversion ratio, mineral retention in breast muscle and liver, fecal mineral excretion and digestibility of minerals when compared to unsupplemented diets (Table 12).

5. Discussion

"Micronutrient malnutrition is a global problem and it can lead to several metabolic and pathophysiological disorders, which are potential consequences of failure to ingest and to absorb sufficient amounts of essential or beneficial trace elements" (House, 1999). One of the best ways to overcome this problem is to increase levels of micronutrients in staple food such as cereals.

There are two ways to improve cereals as dietary source of essential micro-minerals. First one is to enhance the absorption and utilization of minerals by increasing their quantity in cereals and second one is to decrease the quantity of phytate and some other anti-nutrients that inhibit micro-mineral absorption in intestines (Welch and Graham, 2004). Mostly just one of these methods is used. In this experiment both of them were combined in order to increase iron and zinc levels in chicken meat.

In the initial stage of this experiment, when wheat varieties Srpanjka and Simonida were selected for feed production it was considered that levels of iron and zinc are significantly lower in these varieties. This assumption was based on some previous analyses performed on them. It was also expected that chickens should develop symptoms of deficiency and that phytase addition will enhance mineral absorption due to the fact that dietary levels of these minerals are low. However, ICP-MS analyses showed that feed with wheat as only dietary source of iron and zinc contained high levels of these minerals. Contrary to our initial opinion, not only diets with wheat biofortified with zinc but also diet with wheat biofortified with selenium had sufficient amount of iron and zinc. Analyses also showed difference between

iron and zinc levels in the same diet with and without phytase, but this is due to the variation in analytical accuracy of analyses and differences were not statistically important.

The analyses of feed also shows that wheat biofortified with zinc has higher levels of iron compared to wheat biofortified with selenium. This suggest that increased levels of zinc may bind more phytate, what leaves additional space for iron absorption in plants.

5.1. Broiler performance

"Growth response in broiler chickens has been used as the primary criterion for determining requirements of trace minerals because broilers are ideal assay animals with a limited nutrient store, high nutrient demand and rapid growth rate" (Baker and Ammerman, 1995; Bao et al., 2009). Requirement for early broiler growth is satisfied when broilers are fed on diets containing 80 mg/Fe and 40 mg Zn/kg (NRC, 1994).

Despite the variation in zinc levels (37 to 57 mg/kg) and iron levels (140 to 240 mg/kg) there was no improvement in total body weight or feed intake. This may be explained by the fact that body can absorb only restricted amount of minerals and that excessive intake of minerals will lead to increased mineral excretion and not increased absorption. All diets in this experiment had sufficient levels of these minerals and even diet with lowest level of iron and zinc is not showing deficiency. In the study of Mohanna and Nys, (1999), it was found that increase in body weight and feed intake can only be observed until supplementation with 45 mg/kg total dietary Zn was reached and that there was no additional response at higher zinc concentrations. Collins and Moran (1999) also confirmed in their studies that increased concentrations of dietary zinc have no effects on broiler live performance.

Ma et al., (2012) showed that when it comes to the iron requirements optimal growth (651 g/bird) is achieved when there is 120 mg/kg of Fe in feed.

Commercial grower diet which was control diet in this experiment contained 190 mg/kg of Fe and 93 mg/kg of Zn. This level of mineral supplementation is much higher than recommended one, which leads to conclusion that producers are using mineral supplements with a large safety margin.

The average weight gain on the control diet in our experiment was 669 g/bird, and it can be concluded that increase in mineral supplementation above the recommended levels will not lead to significant weight gain.

Average weight of broilers fed with experimental diet at 21 day of age ranged between 872-962 g and there was no significant difference between diets ($P > 0.05$). The difference between all experimental diets and control diet was very significant ($P < 0.001$). It may be assumed that depressed feed intake and body weight gain in broiler chickens fed with control diet is due to the extensive manipulation with this diet. Control diet was purchased in the form of pellets which means that it was exposed to numerous changes, including heat treatment. To avoid differences in feed appearance pellets were grinded. Grinded pellets had a finer structure compared to other diets and chickens probably spilled this diet more and had more difficulties eating this diet.

Increase in weight gain was proportional to increase in feed intake.

Average feed conversion ratio (FCR) reached in experimental diets was significantly lower ($P < 0.001$) then the one in control diet and also contrary to other experiment observation (Ma et al., 2012; Bao et al., 2007; Mohanna and Nys, 1999). The reason for this is possibly due to poor consumption of control diet, low growth rate of chickens fed with control diet and feed spillage.

5.2. Mineral content in liver and breast muscle

Tissue mineral concentration data are usually used to evaluate mineral status of animals and humans (Feng et al., 2009). This study showed no significant effect of different diets on concentration of iron and zinc in breast muscle and liver. This is contrary to several studies who demonstrated increase in breast muscle and liver iron concentration with increase in dietary iron, with significant level ($P = 0,001$) (Cao et al., 1996; Ma et al., 2012).

Bao et al., (2007) reported increased concentration of iron and zinc in the liver of birds on control diet ($P < 0.05$) when compared with those on the supplemental treatment, but not among different supplemental treatments. Control diet in Bao et al. (2007) study was made to meet or exceed broilers requirements.

Average iron and zinc concentration in breast muscle in the study of Ma et al., (2012) were ranging between 8.54 – 11.31 mg/kg for iron and 0.54 – 0.56 mg/kg for zinc, while our average data varied between 3.5 – 4.5 mg/kg for iron and 6 – 7.1 mg/kg for zinc for 21 day old chickens. On the other hand, average iron and zinc concentrations in the liver in their studies were between 121 – 145 mg/kg for iron and 64.4 – 64.8 mg/kg for zinc, and in our studies it was between 162 – 253 mg/kg for iron and 24.3 – 28.6 mg/kg for zinc. Both studies

gave absolute opposite results. It can be assumed that average intake of iron in feed will enhance iron levels in breast muscle, while excessive intake of iron is enhancing iron levels in liver. Too high intake of zinc will enhance zinc deposition in liver.

5.3. Mineral excretion

Although there is no statistically important difference among experimental diets it can be observed that there is a linear response to concentrations of fecal iron with the increasing levels of dietary iron. Results of study by Creech et al. (2004) showed decreased level of fecal iron in piglets fed with reduced levels of iron in the diet, whereas increased dietary Fe level resulted in a linear increase of fecal iron excretion.

It is also observed in our study that broilers on diet with wheat Srpanjka and Simonida (Se2 soil) - with phytase, which was supplied with lowest levels of iron (140mg/kg) and zinc (37 mg/kg), supported best FCR, had highest amount of deposited iron in breast muscle and liver and highest amount of deposited zinc in breast muscle and showed lowest iron and zinc fecal excretion. This diet obtained statistically important difference in iron ($P < 0.01$) and zinc ($P < 0.001$) fecal excretion when compared with control diet. This can be explained with the fact that chickens are able to compensate lower levels of iron and zinc in the diet by increasing their absorption in intestines.

The excretion of zinc increased ($P < 0.001$) linearly with increasing intakes of this mineral. This clearly suggests that the highest levels of zinc used in this study do not contribute to mineral deposition in tissues but are excreted. It confirms that changes in zinc absorption and excretion in the gastrointestinal tract are the primary mechanisms for maintaining zinc homeostasis (King et al., 2000).

Our study confirmed results of Bao et al. (2007) and Dozier et al. (2003) study which indicated that the highest trace mineral supplementation had no additional effects on broiler performance and it is possible to use lower levels of trace mineral supplements without compromising bird growth or increasing the rate of excretion.

5.4. Digestibility of iron and zinc

Digestibility values for iron were greatly affected by its concentration in diets. Digestibility of iron decreased with the decreasing intake in feed. With that been said, it is obvious from Table 11 that lowest digestibility was in diet with wheat Srpanjka and Simonida (Se2 soil) -

with phytase, with statistically significant difference when compared with diets with wheat Srpanjka (ZnSO4) – with phytase, wheat Simonida (ZnSO4) - with phytase, wheat Srpanjka (ZnSO4) - no phytase and wheat Simonida (ZnSO4) - no phytase. Diet with wheat Srpanjka and Simonida (Se2 soil) - no phytase also had significantly lower digestibility ($P < 0.05$) when compared with diets with wheat Simonida (ZnSO4) - with phytase and diet with wheat Simonida (ZnSO4) - no phytase that show highest digestibility.

Apparent digestibility of iron in this study ranged between 20 and 40 % depending on the diet. This confirms results of the Vandeberg and De La Noue, 2001 study in which apparent digestibility is 27 % when using column method of feces collection. In the study of Ettle et al. (2008) digestibility of iron in piglets was between 30.7 and 40.9 %.

Zinc had negative digestibility values in the feces of chickens fed with diet with wheat Simonida (ZnSO4) - with phytase . This result is probably consequences of high amount of this mineral in the diet and restricted possibility of chickens to absorb this mineral in intestines. However, Vandeberg and De La Noue, 2001 discussed in his study that negative digestibility can be due to trace amounts of blood contaminating the feces, so it is not possible to exclude this reason as an explanation for negative digestibility in our study. Highest digestibility (40 %) is unlike iron digestibility observed in diet with wheat Srpanjka and Simonida (Se2 soil) - with phytase which had lowest supplementation of zinc. Apparent digestibility of zinc in Vandeberg and De La Noue, (2001) was 59.4 %, and that is obviously higher compared to result in this experiment.

Results indicate that diets with lowest levels of iron and zinc (Wheat Srpanjka and Simonida (Se2 soil) with and without phytase) had lowest digestibility of iron and highest digestibility of zinc. High digestibility of zinc can be explained as previously sad with the fact that chickens are trying to compensate low levels of mineral in the diet with increased digestibility. The reason why these diets had lowest iron digestibility is not possible to explain for now.

5.5. Influence of phytase on broiler performance

In our study there was no significant effect ($P > 0.05$) of phytase addition on weight gain, feed intake, feed conversion ratio, mineral retention in breast muscle and liver, fecal mineral excretion and digestibility of minerals when compared to unsupplemented diets. Availability of iron and zinc can decrease when higher levels of phytic acid are included in diet, because

phytic acid has ability to form complexes with cations such as iron and zinc (Vohra et al., 1965). That is the reason way, with the addition of enzyme phytase, iron and zinc availability should increase. However, because that did not happen in our experiment, it indicates that phytase has no affect when iron and zinc are nutritionally adequate in the diet. It can be concluded that lack of effect happened because organism is modifying the rate of gastrointestinal absorption of minerals according to body needs.

The lack of significant effect of phytase addition was also observed in the study of Wiliams et al., (2005) were phytase did not affect growth performance of pigs regardless of the level of dietary zinc. Phytase supplemented at 1500 and 3000 U kg^{-1} did not significantly ($P > 0.05$) improve the iron retention in 21 day old broiler chickens (Chung et al., 2013).

This is contrary to various other studies, where addition of phytase to the diets has been consistently shown to enhance growth performance. Simons et al. (1990) reported that phytase addition (1500 FTU kg−1) to diets improved weight gain of broilers from 338 to 733 g and improved feed conversion ration coefficient from 1.85 to 1.5. In the studies performed on broilers from 7 to 25 days of age, Selle et al. (1999) found that addition of 600 FTU kg−1 phytase will increase weight gain (7.6 %) and feed efficiency (4.7 %), while Cabahug et al. (1999) reported that phytase addition 400 and 800 FTU kg−1 to the diets increased weight gain (18.8%), feed intake (9.0%) and feed efficiency (7.9%).

Cabahaug et al., (2010) found that the maximum responses in body weight gain and food efficiency to supplemental phytase, was achieved by the inclusion rate (400 FTU/kg) at all dietary levels of phytic acid and additional supplementation had marginal improvement.

In the study of Cowieson et al. (2004) the ingestion of 1 g of phytic acid by broilers increased the excretion of endogenous nitrogen, amino acids, iron, sodium, sulphur and sialic acid. Supplementation of phytic acid with exogenous phytase reduced the excretion of endogenous amino acids, calcium, sodium, phytate phosphorus and sialic acid compared with birds fed with phytic acid.

6. Conclusion

In conclusion, the results of this study indicate that wheat Simonida and Srpanjka, when it comes to the broiler chicken requirement, have adequate dietary level of iron and zinc and that it is not necessary to add any additional supplement to the feed that contains any of this wheat varieties.

Diet that contained wheat Simonida and Srpanjka without any zinc treatment during crop production and with phytase addition showed best results. The reason for this lays in the fact that this diet had lowest but still sufficient amount of iron and zinc and therefore it had lowest excretion in feces and highest absorption in intestines. From this we can conclude that dietary levels of 120 mg Fe/kg diet and 37 mg Zn/kg diet can be considered as optimal for broiler chickens.

Phytase was not effective in improving iron and zinc availability, due to excess amounts of this minerals in all diets.

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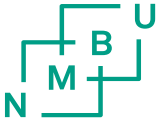

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