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Is the Amount of L-carnitine and Methionine-Lysine Affect on the Microbial Flora of Broiler Cecum?

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This experiment investigates the effects of different levels of L-carnitine and additional lysine-methionine levels on the intestinal microflora of Ross 308 male broilers. Experiment was based on 3×3 completely randomized factorial design. Different levels of L-carnitine including; 0 (mg/kg), 75 (mg/kg) and 150 (mg/kg) and additional lysine-methionine levels were selected from the recommended NRC levels including 0%, 15% and 30%. One bird from each replicate was selected and slaughtered at the age of 42 days; furthermore, cecum was separated and measured to determine the microbial flora. The results showed the levels of L-carnitine caused a significant difference upon the total population of aerobic bacteria, producing lactic acid bacteria, *Escherichia coli* and lactobacilli ($p<0.05$). There was no significant difference in the parameters at all levels. Moreover, additional methionine-lysine levels caused a significant difference in total population of aerobic bacteria and coliforms ($p<0.05$). Finally, the interaction of L-carnitine and lysine-methionine in intestinal microflora caused a significant difference in total population of aerobic bacteria, producing lactic acid bacteria, *Escherichia coli*, and lactobacillus. There was no significant difference in the parameters at all levels.

Key words: L-carnitine, Lysine-methionine, Broiler chickens, Intestinal microflora.

According to increasing the population and results in the meat consumption, it is estimated demand will be166 million tons for the next half-century. For this purpose, the average of production rates should annually increase by 4.5%, while growth in poultry production was 4% in the past half-century. In fact, the demand has outstripped the supply. More research is needed to increase poultry production to meet human needs.

Nowadays, L-Carnitine is utilized as a supplement in poultry nutrition[1, 2]. L-Carnitine is a vitamin-like in human and animal's body due to its role in the energy metabolism. In this regard, its supply is necessary in the body.

For the first time, Glowich and Kimbrider have separated the L-carnitine from muscle tissue and then characterized its important role in the metabolism of fats and carbohydrates. Furthermore, it is required for the proper functioning of the heart muscle.

Due to the discovery of L-carnitine from muscle tissue, the name originated from the "Caro" and "Caris" which means the meat. In humans and animals, L-Carnitine primarily synthesized in the liver and transported to the muscles. About 98% of the sources of L-carnitine are in skeletal and heart muscle.

On the other hand, L-carnitine is also absorbed from food and however, L-carnitine is always a mixture of both sources (food source and synthesized in the body)in the body. Carnitine synthesis requires essential amino acid such as lysine and methionine and also traces nutrients as

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well as iron, vitamin C and B_6 vitamins. Though, the lack of trace nutrients, resulting in decreased synthesis of L-Carnitine and muscle fatigue.

Due to the dependence of trace nutrients, L-carnitine has been studied as a vitamin-like cholinesterase alpha lipoleic acid[3-8].

Research also shows that the relation between semen characteristics (volume, rate, motility) and the L-carnitine of diet in cockerel. Studies have shown L-carnitine is highly effective in increasing sperm count, concentration and, motility.

Research on young and old cockerels showed that cockerels fed by supplementation containing L-carnitine increased the sperm count and better response to stress. Therefore, L-carnitine is recommended to increase sperm fertility[9,10].

methionine deficiency decreases body weight gain of broilers during the first 6 weeks[11]. In turkey chickens, deficient methionine and adequate cysteine in diets was led to swelling in the foot pad.

lysine deficiency causes growth retardation and reduced levels of protein metabolism in the liver[12]. lysine deficiency causes the turkey wing feathers to be discolored. lysine deficiency reduced blood hemoglobin and hematocrit.

lysine is considered as an essential amino acid however, it must be supplied through diet for poultry. Since, 50 to 60 percent of the poultry diet is dependent on grains (poor lysine and methionine);therefore, its value is related to supply the lysine content. In the previous literatures have been studied the broilers lysine and its dependence on the diet protein and metabolisable energy (ME) in three periods; beginning, growth and ending[11, 13].

Accordingly, this experiment investigate the effects of different levels of L-carnitine and excess lysine-methionine levels on the intestinal microflora of Ross 308 broilers.

MATERIALS AND METHODS

In this experiment, 9 treatments were used with 3 replicates and each replicate includes 10 birds in each pen. 270 pieces of one-day old male Ross 308 Broiler chickens were transferred into 27 pens measuring 5.1×1 square meters.

Experimental period was 42 days, which includes; 21-day beginning, 14 days the growth, and 7 days ending periods. It should be noted that all one day male chicks were directed sex-determination and then randomly divided into the experimental units.

Experimental treatment

Experimental design used in this study was based on 3×3 completely randomized factorial design with 9 treatments and 3 replications, which was set up to investigate the effect of different levels of supplemental L-carnitine and excess lysine-methionine.

Treatments were as follow:

1. Control (basal diet)
2. The second group consisted of basal diet with 15% lysine-methionine more than the NRC recommendation
3. The third group consisted of basal diet with 30% lysine-methionine more than the NRC recommendation
4. The fourth treatment consisted of basal diet with 75 mg/kg supplemental L-carnitine
5. The fifth treatment consisted of basal diet with 75 mg/kg supplemental L-carnitine and 15% lysine-methionine more than NRC recommendation.
6. The sixth treatment consisted of basal diet 75 mg/kg supplemental L-carnitine and 30% lysine-methionine more than NRC recommendation.
7. The seventh treatments included basal diet with 150 mg/kg complements L-carnitine
8. The eighth treatments included basal diet with 150 mg/kg complements L-carnitine and 15% lysine-methionine more than NRC recommendation.
9. The ninth treatment included basal diet with 150 mg/kg complements L-carnitine and 30% lysine-methionine more than NRC recommendation.

Intestinal microflora

The experiment was done on poultry farming and Agricultural Sciences and Laboratory of Microbiology of Islamic Azad University, Rasht Branch, Iran. To perform this trial, one chick was randomly selected and slaughtered from each treatment on the final day. After separation of the carcass, the cecum was removed and sent to the laboratory through sterile plates.

A powder of trademark owned by Merck was used for the preparation of the cultures of intestinal microflora. For bacterial growth and its colony counts, the media was primarily prepared according to the guidelines of Merck 2010 catalogue.

Microbial parameters measured in this study were (1) the total population of aerobic bacteria, (2) the population of *Escherichia coli*, (3) the population of coliforms bacteria, (4) the population of lactobacilli bacteria, (5) the population of lactic acid-supplier bacteria, and (6) the population of Enterococci.

Process before microbial culture

Peptone Water preparation

The powder is used for dilution of cecal contents. Some powder (5.25 gr/lit) was poured into the Erlenmeyer flask and then distilled water was added into the flask according to the instructions written on the container. Flask was shaken until the powder dissolved in water. Then, it was placed upon the hot plate to purify and transparency. After preparation of the flasks containing peptone water, 9 ml peptone water was removed from the flasks and poured into a test pipe. Then, it was placed in the autoclave for sterilization by the temperature of one hundred and twenty-one Celsius and one atmosphere for fifteen to twenty minutes. For each sample, nine pipes containing 9 ml of solution was required that totally, 243 pipes were prepared for experiment.

MacConkey Agar preparation (Solid culture medium)

This culture medium represents coliforms populations. A little of culture medium powder (50 g/liter) was poured into the flask and mixed with distilled water.

Flask was shaken until the powder being dissolved in water. To purify and transparency, it was placed on a hot plate.

Then, it was placed in the autoclave for sterilization by the temperature of one hundred and twenty-one Celsius and one atmosphere for fifteen to twenty minutes.

It removed out of the autoclave in order to be completely cool. After cooling, medium was transferred to a sterile plate. It must be considered the location where the plates are placed, must be thoroughly cleaned with alcohol and cotton.

A.M.B culture medium preparation (Solid culture medium)

This culture medium represents E .coli populations

A little of culture medium powder (36 g/liter) was poured into the flask and mixed with distilled water.

Flask was shaken until the powder being dissolved in water. To purify and transparency, it was placed on a hot plate.

Then, it was placed in the autoclave for sterilization by the temperature of one hundred and twenty-one Celsius and one atmosphere for fifteen to twenty minutes.

It removed out of the autoclave in order to be completely cool. After cooling, medium was transferred to a sterile plate. It must be considered the location where the plates are placed, must be thoroughly cleaned with alcohol and cotton.

Nutrient Agar culture medium (Solid culture medium)

This culture medium represents Aerobic bacteria populations

A little of culture medium powder (20 g/liter) was poured into the flask and mixed with distilled water.

Flask was shaken until the powder being dissolved in water. To purify and transparency, it was placed on a hot plate.

Then, it was placed in the autoclave for sterilization by the temperature of one hundred and twenty-one Celsius and one atmosphere for fifteen to twenty minutes.

It removed out of the autoclave in order to be completely cool. After cooling, medium was transferred to a sterile plate. It must be considered the location where the plates are placed, must be thoroughly cleaned with alcohol and cotton.

M.R.S agar culture preparation (solid medium)

This culture medium represents lactic acid supplier populations A little of culture medium powder (2.68 g/liter) was poured into the flask and mixed with distilled water. Flask was shaken until the powder being dissolved in water. To purify and transparency, it was placed on a hot plate. Then, it was placed in the autoclave for sterilization by the temperature of one hundred and eighteen Celsius and one atmosphere for fifteen to twenty minutes.

It removed out of the autoclave in order to be completely cool. After cooling, medium was transferred to a sterile plate. It must be considered the location where the plates are placed, must be thoroughly cleaned with alcohol and cotton.

Rogosa Agar culture medium preparation (Solid medium)

This culture medium represents Lactobacillus populations

A little of culture medium powder (5.74 g/ liter) was poured into the flask and mixed with distilled water.

Flask was shaken until the powder being dissolved in water. To purify and transparency, it was placed on a hot plate.

Then, it was placed in the autoclave for sterilization by the temperature of one hundred and twenty-one Celsius and one atmosphere for fifteen to twenty minutes

It removed out of the autoclave in order to be completely cool. After cooling, medium was transferred to a sterile plate. It must be considered the location where the plates are placed, must be thoroughly cleaned with alcohol and cotton.

Azide Agar culture medium preparation (Solid culture medium)

This culture medium represents Enterococci populations

A little of culture medium powder (65.54 g/liter) was poured into the flask and mixed with distilled water.

Flask was shaken until the powder being dissolved in water. To purify and transparency, it was placed on a hot plate.

Then, it was placed in the autoclave for sterilization by the temperature of one hundred and twenty-one Celsius and one atmosphere for fifteen to twenty minutes.

It removed out of the autoclave in order to be completely cool. After cooling, medium was transferred to a sterile plate. It must be considered the location where the plates are placed, must be thoroughly cleaned with alcohol and cotton.

Cultivate of microbial flora cecal

After preparation of peptone water and media, for each cecum sample and from each culture medium respectively, 9 peptone water pipe, and3 medium was removed (Eighteen medium for each cecum). Totally, for 27 samples of the cecum, 243 pipes containing 9 ml peptone water and 486 plate containing culture medium was considered. Peptone water was used for dilution of the cecal contents.

At first, one gram of cecal contents of chickens was separated with a sterile scalpel and put into a sterile plate. For each cecum, 9 pipes were removed and numbered from one to nine.

In the first step, the total solution of 1st pipe were poured into a pipe containing cecal contents and stirred to obtain a homogeneous mixture.

Then, by Sampler, thousands micro lambda compliance with sanitation, solution with the number one picked up and transferred to the pipe number two and serial dilution were accordingly done until number nine.

Three samples were selected from each culture, marked behind by a pen and were separately addressed by the names and numbers.

After dilution of the cecal contents by peptone water, tubes number five, seven, nine were selected and one hundred micro lambda was picked up by sampler from each tube and then, evacuated into the culture based on matching the number of tube and culture plate (e.g. we decanted separately 100 lambda from tube number five onto the plate of the Azide 5, Macconkey 5, nutrient 5, Rugasa 5, M.R.S)

After discharging the solution upon the culture medium by sterile tube, the tubing, the liquid was spared on the entire plate.

Finally, the culture medium transferred into the incubator after putting the lids. The incubator was set to 35 degrees in order to the colonies were forming after 2-4 days; it is noteworthy that, for convenience, plates were incubated upside down (From the door side). After the colony formation, counts of bacteria were made by direct observation and manual.

Statistical analysis

Experiment was based on 3×3 completely randomized factorial design (3 L-carnitine and 3 lyine-methionine levels).As a result, the experiments consisted of nine treatments and three replicates of each treatment and the model is designed as follow:

$$X_{ijk}=\mu+\alpha_j+\beta_k+(\alpha\beta)_{jk}+\varepsilon_{ijk}$$

μ = Population effects average

α_j = effect of factor A (L-carnithine level)

β_k = effect of factor B (methionine-lysine level)

$(\alpha\beta)_{jk}$ = AB interactions
ε_{ijk} = the experiment error

After measuring different variables, these values entered into the computer through EXCEL software and statistical analysis was performed by SAS software. Statistical data variables including analysis of variance and means comparison was performed through Duncan's method.

Experiment was done in accordance with the legal requirements of the relevant local and national authority.

RESULTS

Obtained results are summarized in Tables 1-3.

Effects of different levels of L-carnitine upon some populations of cecum bacteria

We concluded from the effect of different levels of L-carnitine on the population of the cecum bacteria that the levels of L-carnitine 0 mg/kg, 75 mg/kg and 150 mg/kg caused a significant variance on total aerobic bacteria, lactic acid bacteria, *Escherichia coli* bacteria, Lactobacilli, coli forms ($p<0.05$).

The total aerobic and lactic acid bacteria had numerically higher amount at 75 (mg/kg) levels and lowest amount at 150 mg/kg levels. j

For *Escherichia coli* bacteria and coliforms, the levels of 0 and 150 mg/kg are the lowest and highest amounts respectively, in terms of the numbers. Finally, for lactobacilli, the levels of 150 mg/kg are the lowest and 75 mg/kg the highest in terms of the numbers. Results were not significant on Enterococci population.

Effects of additional lysine-methionine levels over the populations of some cecal bacteria

According to the results obtained from the table that illustrates the effect of additional lysine-methionine over the populations of cecum bacteria, we conclude that the effect of lysine-methionine levels such 0, 15 and 30% led to a significant difference in the total population of aerobic bacteria and coliforms ($p<0.05$).

For the all aerobic bacteria in terms of amount the level of null and 15% are lowest and highest respectively. Coliforms in terms of number at the level of 15% and 30% are the lowest and highest respectively. Results have shown no significant difference over the supplier lactic acid

Table 1. Comparison of the population average of cecum bacteria (± SD means) under different levels of excess L–carnitine.

L-carnitine Microbial population	0(mg/kg)	75(mg/kg)	150(mg/kg)	Sig	SEM
aerobic bacterial populations (Colony forming units/g)	313857143 ^{a}±224288080	686000000 ^{b}±892742964	286571429 ^{b}±82376892.28	**	13385868 3
lactic acid-producing bacterial populations (Colony forming units/g)	93285714 ^{a}±28429528.58	40400000 ^{b}±24016660.88	92000000 ^{a}±22500793.64	**	6556967.8
Escherichia coli populations (Colony forming units/g)	42333333 ^{b}±9287985.07	102142857 ^{a}±88578455.94	113833333 ^{a}±51281250.63	**	7922399
General population j form (Colony forming units/g)	69000000 ^{b}±24347484.47	140000000 ^{ab}±138121927	174000000 ^{a}±106622699	*	17623404
Lactobacillus populations(Colony forming units/g)	29750000 ^{b}±24875244.28	76500000 ^{a}±50295791.74	50555556 ^{ab}±7762087.35	*	6674127.8
Enterococcus populations (Colony forming units/g)	3500000 ^{a}±2428991.56	2266667 ^{a}±1155277.74	5142857 ^{a}±2794552.52	ns	710966.42

*, ** and ns are significant at the levels of 5% and 1% and non-significant respectively. Also, in each row, the same letters indicate no significant differences

Table 2. Comparison of the population average of cecum bacteria (± SD means) under different levels of excess lysine-methionine.

lysine-methionine Microbial population	+0%	+15%	+30%	Sig	SEM
aerobic bacterial populations (Colony forming units/g)	182444444 ^{b}±73065229/61	971833333 ^{a}±916811958	316857143 ^{b}±77565947.36	**	133858683
lactic acid-producing bacterial populations (Colony forming units/g)	86833333 ^{a}±35079433.67	97400000 ^{a}±19256167.84	64777778 ^{a}±34302980.11	ns	6556967.8
Escherichia coli populations (Colony forming units/g)	75714286 ^{a}±61662290.71	87750000 ^{a}±44589797.04	96375000 ^{a}±82961157	ns	7922398.8
General population j form (Colony forming units/g)	102333333 ^{b}±59815271.18	76714286 ^{b}±62914530.08	214500000 ^{a}±141396959	**	17623404
Lactobacillus populations(Colony forming units/g)	46500000 ^{a}±23441416.34	65400000 ^{a}±45768985.13	45666667 ^{a}±30572318.63	ns	6674127.8
Enterococcus populations (Colony forming units/g)	3000000 ^{a}±2738612.79	4800000 ^{a}±2523885.89	3142857 ^{a}±2193062.66	ns	710966.42

*, ** and ns are significant at the levels of 5% and 1% and non-significant respectively. Also, in each row, the same letters indicate no significant differences.

Table 3. Comparison of the population average of cecum bacteria (± SD means) under different levels of excess L–carnitine and lysine-methionine.

Treatment Microbial population	1	2	3	4	5	6	7	8	9	Sig	SEM
aerobic bacterial populations(Colony forming units/g)	2.08×10^{8b}± 32192132	4.75×10^{8b}± 459619408	3.12×10^{8b}± 52325902	$1\times10^{7\ b}$± 37634204	2.1×10^{9a}± 431335137	3.33×10^{8b}± 84551759	2.4×10^{8b}± 51961524	3.46×10^{8b}± 64346717	2.98×10^{8b}± 132228968	**	231850041
lactic acid-producing bacterial populations (Colony forming units/g)	9.5×10^{7ab}± 48790368	1.1×10^{8a}± 21920310	8.0×10^{7abc}± 18583146.5	3.6×10^{7c}	8.2×10^{7abc}	2.8×10^{7c}± 6928203.2	9.9×10^{7ab}± 18036999	9.0×10^{7abc}± 14142135.6	8.7×10^{7abc}± 35118845.8	*	11357001
Escherichia coli populations (Colony forming units/g)	5.3×10^{7bc}± 4242640	3.7×10^{7c}±	3.7×10^{7c}± 6000000.0	$3.2\times10^{7\ c}$± 7637626.2	6.4×10^{7bc}	$1.9\times10^{8\ a}$± 70152215	1.65×10^{8a}± 7778174.6	1.3×10^{8ab}± 7071067.8	5.2×10^{7bc}± 2828427.1	**	13721997
General population j form (Colony forming units/g)	9.1×10^{7c}± 20506097	4.4×10^{7c}± 5656854	7.3×10^{7c}± 17677670	4.4×10^{7c}± 22627417	5.1×10^{7c}± 13435029	$2.6\times10^{8\ ab}$± 129307128	1.7×10^{8bc}± 4949747	1.2×10^{8bc}± 87726849	$3.5\times10^{8\ a}$	**	30524631
Lactobacillus populations (Colony forming units/g)	4.3×10^{7ab}± 25166114	4.0×10^{7ab}± 19078784	6.8×10^{7ab}± 27537852.7	8.0×10^{7ab}	1.04×10^{8a}± 53033008	1.9×10^{8b}	3.5×10^{7ab}± 6363961.0	-	$2.5\times10^{8\ b}$± 7071067.8	*	11559928
Enterococcus populations (Colony forming units/g)	3.5×10^{6a}± 353553	4.0×10^{6a}± 282842	3.0×10^{6a}± 293341	1.0×10^{6a}	3.3×10^{6a}± 848528.14	2.0×10^{a}± 100000	3.5×10^{6a}± 345571	6.3×10^{6a}± 422798	5.0×10^{6a}± 612941	ns	1231429.9

*, ** and ns are significant at the levels of 5% and 1% and non-significant respectively. Also, in each row, the same letters indicate no significant differences.

bacteria, *Escherichia coli*, Lactobacillus and Enterococci for all levels of lysine-methionine as compared to other experimental groups.

Effects of L-carnitine and additional lysine-methionine levels over the populations of some cecal bacteria j

According to the results obtained from the table that illustrates the effect of additional lysine-methionine over the populations of cecum bacteria, we conclude that the effects of treatments caused significant difference in the overall population of aerobic bacteria, lactic acid-producing bacteria, *Escherichia coli*, Lactobacillus, and coliforms ($p<0.05$).

Totally, the aerobic bacteria of first treatment have the lowest and the fifth treatment the highest amount in terms of number. Lactic acid-producing bacteria of sixth treatment have the lowest and second treatment have the highest in terms of number. Coliforms of second treatment have the lowest and ninth treatment have the highest in terms of number. Lactobacillus of second treatment has the lowest and ninth treatment has the highest in terms of number. The effect of treatments had no significant difference over the populations of enterococci ($p>0.05$).

DISCUSSION

In an overall view on the intestinal microflora, any increasing factor in the population of aerobic bacteria, lactic acid-producing lactobacilli and Enterococci ends up to health improvement, reduce bird activity, mediate pathogenic microflora populations and finally, increasing the quality and quantities related to production. Any reduction in the population of coliforms and *Escherichia coli* in the same way makes it possible to achieve the same goal.

Coliforms

The results have shown the coliforms were affected by different levels of L-Carnitine and additional levels of lysine-methionine. The ninth treatment was considered as the worst and the second as the best. Coliform can be used as an indicator for the quality of food and water. Coliform bacteria are Gram-negative, non-spore, anaerobic, which can ferment the lactose with the production of acid and gas at a temperature of 35 to 37 Celsius. When the total number of coliforms reaches to more than normal, it can cause some diseases in humans and animals. Coli form presence may use for determination of the other pathogenic organism disease.

Escherichia coli

The results shows *Escherichia coli* bacteria levels were affected by different levels of L-carnitine but an extra level of lysine-methionine have not significantly affected upon *Escherichia coli* population. Fourth treatment was the best and the sixth the worst.

Escherichia coli is a Gram-negative, spherical shape, and optionally anaerobic and non-spore bacteria. The harmless strains of bacteria form the microbial flora of digestive tract. As a result, it can produce vitamin K_2 to prevent establishment of pathogenic bacteria in gut.

Lactobacillus and lactic acid-producing bacteria

The results showed Lactobacillus, lactic acid-producing bacteria were affected under different levels of L-carnitine but the effects of additional lysine-methionine over the producing lactic acid bacteria and Lactobacillus population was not significantly different. The ninth treatment was the best and the second was the worst.

Lactobacillus acidophilus is considered as a ferm enter which it ferments the sugar into lactic acid. These bacteria exist naturally in the digestive tract of humans and animals and some strains are probiotics. Acid production by Lactobacillus has beneficial effects on the fungal contamination in this area (Lyjvnq *et al*, 2006)[16].

Lactobacillus strains consider as a measure to improve the health of the intestinal microflora and cause it to be considered as mechanisms that inhibit pathogen: competition for colony formation, the race for food, the competition to stimulate the immune system, lactocin production, acidoline with gram-negative and acidophylus with gram-positive inhibition[14].

Enterococci

The results showed that Enterococci were not affected under different levels of L-carnitine and excess levels of lysine-methionine. Enterococci are classified as optional anaerobic and do not produce spores. An important genus of gram-positive cocci is seen as the pair (diplococci) or short chains. From the medical point of view, Enterococci are resistance and inherent antibiotics[15].

Aerobic bacteria

The results showed that L-carnitine and additional lysine-methionine levels caused significant difference on the total population of aerobic bacteria. First and fifth treatments were the best and the worst treatments respectively.

Finally, the interaction of L-carnitine and lysine-methionine at this study were caused significant difference over the total population of aerobic bacteria, producing lactic acid bacteria, *Escherichia coli*, Lactobacillus, and coliforms, but it had no significant difference over the enterococcus populations.

CONCLUSION

According to the positive effects of L-carnitine on the microflora of the cecum that was led to a dramatic improvement of vital parameters associated with the health of the birds and also the positive and constructive role and often synergistic lysine-methionine more than the recommended NRC in helping to improve the numerical parameters are critical in the broilers in this study, it is recommended to use the values of 150 (mg/kg) L-carnitine and 15% lysine-methionine more than NRC recommendations, in broiler chicken diets.

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