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# The antimicrobial activity of neutral electrolyzed water against germs and fungi from feedstuffs, eggshells and laying hen house

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## Abstract

*The work consisted in studying the sanitation effect of neutral anolyte(ANK) on pathogen agents from poultry housing space, eggshells and feedstuffs, used to laying hens nutrition. It has used three solutions of neutral anolyte in water: a)ANK solution with an active chlorine content of 8mg/l and 12mg/l respectively, used as drinking water for laying hens; b)ANK solution with an active chlorine content of 8mg/l and 12mg/l respectively used for treatment the seven types of feedstuffs (maize, barley, maize gluten meal, rice, barley, soybean meal and canola meal), three mixed feeds, and five types of eggshells, respectively. Treatments consisted in the immersion for 15min of each sample in ANK solution; c)ANK solution with an active chlorine content of 36mg/l used for hen-house space treatment by washing it for 15 min. The ANK solution with 36 mg/l active chlorine had a total bactericidal effect on the Total coliform, meantime the total number of germ from feedstuffs decreased with 14.29% until to 97% against the control in case of 12 mg/l active chlorine. The experiment highlights the role of active chlorine on the microbiological load in the water, feedstuffs, diets, eggshells and hen-house space. As the concentration of active chlorine is much higher as more significant is the bactericidal effect of the ANK solution.*

**Keywords:** electrolyzed water, Neutral Anolyte ANK, bactericidal effect, electrochemically activated substances

## 1. Introduction

The most important indicators of the laying hen digestive micro flora also in relation with litter contamination level are determined by the bacteriological water properties combined with feedstuffs and mixed diets quality (Hulea Ana et al. [10]). Numerous previous and preventable studies performed, against those aspects with incidental epidemiology cases have highlighted the fact that water is a major provider of pathogen agents with negative effect, given by various bacteria, such as: *Staphylococcus aureus, Escherichia coli, Cryptosporidium, Giardia lamblia, Listeria and Legionella* (Amaral, L. A. [1]; Fenner D. C. et al. [8]). Pathogen agents are simple growing in feedstuffs, in laying house, on cages and on the eggshells also.

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The cleaning effectiveness of slightly acidic electrolyzed water (SAEW) for inactivating the microbes in layer houses was investigated by (Hao X. X. et al. [9]) who showed that SAEW with 250 mg/l active chlorine, pH of 6.19 and ORP of 974 mV, has inactivated 100% of bacteria and fungi in feces and feed. As results of studies performed by scientists from USA (Kim C. et al., [12]; Park C. M. et al. [16]) and China (Wei Cao et al. [18]), the electrolyzed water became a major sanitation product used in food industry. The antimicrobial effect of electrolyzed water solutions in many different food models such as vegetables, meat, poultry, fish was also showed by (Barakat S. M. Mahmoud, [2]). Electrolyzed water can play also an important role in control of pathogen microorganisms from fresh vegetables as: tomatoes, cucumber, lettuce, etc (Deza M.A. et al. [5]). Due to it, low production cost, good bactericidal and ecological properties, in the latest years the electrolyzed water is using on large scale in all kind of animal farm (Fenner D. C. et al. [8]; Jirotkova D. et al. [11]) . Studies performed in order to reduce *Escherichia coli* and *Listeria monocytogenes* from steel or plastic feeding tools and living space for chickens have showed that spraying the surfaces with electrolyzed water (30-50 mg/l active chlorine) has as effect the inactivation of the pathogen agents (Deza M.A. et al. [6]). By using, the electrostatic spraying technique of electrolyzed water on eggs has more efficiency in removing the pathogen agents from eggshell : *Salmonella Typhimurium, Stafilococus aureus, Listeria monocytogenes* (Bialka K. L. et al., [3]; Fasenko G. M. et al. [7]; Russel S.M. [17]). Using, the electrostatic spraying technique of electrolyzed water on broiler carcasses has also more efficiency in removing the aerobic bacteria *Escherichia coli*, *Campylobacter* and *Salmonella* from poultry meat (Northcutt J. et al. [15]). Airborne bacteria from poultry housing space can be effectively inactivated by spraying of membraneless acidic electrolyzed water ( MLAEW) with adequate free chlorine (FC) and contact time. Bacteria were completely inactivated at 0.5 min when samples were treated with MLAEW with FC > 16.8 mg/l, in 2 min when FC > 13.8 mg/l, and in 5 min when FC > 7.2 mg/l (Yang Zhao et al. [19]). The neutral anolyte ANK should be used immediately or in a few days after production in order to prevent the loss of active chlorine that is one of the most important factor contributing to antimicrobial activity (Len S. V. Et al. [13]). Our tests performed in experimental stand with electrolyzed water from INCDBNA Balotesti have as objectives evaluating the effect of electrolyzed water (neutral anolyte ANK) on microbial load from feedstuffs and mixed diets used in laying hen nutrition, from living space, cages and eggshell.

## 2. Materials and Methods

The sample of electrolyzed water has been produced by using an Envirolyte EL 400 unit ([20], [21]). A 25% sodium chloride solution and tap water was simultaneously pumped into unit to obtain neutral anolyte ANK with the following characteristics: pH = 7.42; Oxidation-reduction potential (ORP) = 845 mV, Active chlorine = 445mg/l. The active chlorine quantity from ANK was adjusted to 445 mg/l by keeping the amperage in cell to a value between 23-25 A and the pH was adjusted to 7.40 by reducing/rising the quantity of catholyte evacuated as residue [20]. Solutions with a content of 8mg/l, 12 mg/l and 36mg/l active chlorine were obtained by dilution the ANK with distilled water like: 18 ml ANK and 982 ml distilled water, 27 ml ANK and 973 ml distilled water respectively 81 ml ANK and 919 distilled water. The percentages of ANK in water are 1.8%, 2.7% and 8.1% ANK respectively.

ORP and pH measurements have been performed with YSI Professional Plus instrument. Active chlorine measurements have been performed accordingly to the Envirolyte procedure, as follows:

Method: *Iodine titration*
Protocol:
Anolyte solution 10 ml
$H_2SO_4$ 1N 50 ml
few crystals KI:
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Titration with $Na_2S_2O_3$ x 5 $H_2O$ solution 0.1 N
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Calculation of *active chlorine*, as concentration
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Table nr. 1 – Active chlorine concentration according to volume solution $Na_2S_2O_3 \times 5\ H_2O$ used for titration (ml)

| Volume solution $Na_2S_2O_3 \times 5\ H_2O$ for titration (ml) | Active chlorine concentration (mg/l) |
|---|---|
| 0.58 | 200 |
| 0.85 | 300 |
| 1.40 | 500 |
| 2.00 | 700 |
| 2.10 | 750 |
| 2.25 | 800 |
| 2.40 | 850 |
| 2.50 | 900 |
| 3.00 | 1065 |
| 3.50 | 1240 |
| 4.00 | 1420 |

In order to evaluate the effect of ANK on microbial load from feedstuffs and diets used in laying hen nutrition, from housing space, cages and eggshells, the following samples were taken: I) Feedstuffs (7 samples): wheat, maize, gluten maize, rice, barley, soybean meal and canola in quantity of 250 g /sample. II) Diets for laying hens (3 samples): diet for control lot – MDC, diet for lot E1 – MDE1 and diet for lot E3 – MDE3; III) Eggs samples (four eggs/lot – from five lots) like: "Control lot" (diet MDC) – laying hens that were fed on with tape water. "Level 1 – E1 lot" (diet MDE1) - laying hens that were fed on with ANK solution and a content in active chlorine of 8mg/l. "Level 1 – E3 lot" (diet MDE3) - laying hens that were fed on with ANK solution and a content in active chlorine of 8mg/l. "Level 2 – E1 lot" (diet MDE1) - laying hens that were fed on with ANK solution and a content in active chlorine of 12mg/l. "Level 2 – E3 lot" (diet MDE3) - laying hens that were fed on with ANK solution and a content in active chlorine of 12mg/l. IV) Sanitation samples on the floor (4 samples): two before and two after washing for 15 min the floor with ANK, 36mg/l active chlorine.

On the feedstuffs and diets, samples were performed microbiological analysis for two indicators: *"Total number of germs"* (NTG) and *"Yeast and fungi"* respectively. To determine NTG were used the procedures from standard SR EN ISO 4833-1/2014. To determine *"Yeast and fungi"* were used the procedures from standard SR ISO 21527-1/2009. On the eggs samples were performed microbiological analysis for one indicator: *"Total number of germs"*, according to standard SR ISO 4833-1/2014 and the results were expressed as count cfu/g. On the sanitation samples were performed microbiological analysis for two indicators according to the actually sanitary veterinary regulations like: *"Total number of germs"*, according to standard SR ISO 4833-1/2014, and *"Total coliform bacteria"*, SR ISO 4831- 2006 (Cotrau M. and Maria Proca, [4]; Manescu S. [14]).

All samples were inoculated in Petri dish, doubled, with the dilution of $10^{-1}$, $10^{-2}$, $10^{-3}$ and $10^{-4}$.

The laboratory analysis were performed on four sorts of samples with 2-3 stages for each sort.

*The feedstuffs and diets analysis*

In first stage were performed the initial microbial load for each sample.

In the second stage, each sample composed from 10 g substance was treated 15 min with 90 ml ANK solution containing 8mg/l active chlorine and then were performed tests to determine the two microbiological indicators. In the third stage, each sample composed from 10 g substance was treated 15 min with 90 ml ANK solution containing 12mg/l active chlorine and then were performed tests to determine the two microbiological indicators.

*The eggshell analysis*

In first stage were performed the initial microbial load (NTG) for four eggs from each lot.

In the second stage, two eggs from control lot were immersed for 15 min in 200 ml ANK solution containing 8 mg/l active chlorine than were performed tests to determine the microbiological indicator (NTG).

In the third stage, two eggs from control lot were immersed for 15 min in 200 ml ANK solution containing 12mg/l active chlorine than were performed tests to determine the microbiological indicator (NTG).

*The sanitation tests analysis*

In first stage were performed the microbial load for two samples taken before washing the floor.

In the second stage were performed the microbial load for two samples taken after washing the floor.

In *Total number of germs* have not been included yeast and fungi. The *Total number of germs* are represented by mesophilic aerobic bacteria that were developed on the selective PCA (Plate Count Agar) at a temperature of $30^0$C for 3 days. Yeast and fungi were developed on the selective Yeast Extract Agar DRBC (Agar with chloramphenicoland dichloethane rose Bengal) at a temperature of $25^0$C for three days (for yeast) and five days for fungi.

The removing level of microbial load caused by treatment has been calculated as:

$$Rl_{tr} = (1- \text{Value after treatment/Initial value}) \times 100 \quad [\%]$$

## 3. Results and discussion

*a) The feedstuffs and diets analysis*

Table nr.2 shows the initial value of NTG for feedstuffs and after the treatment applied for 15min with ANK solution containing 8mg/l and 12mg/l active chlorine.

Table nr.2- Removing level of NTG from feedstuffs with ANK solutions

| SAMPLE 10g feedstuff + 90ml ANK | Microbiological indicators | | | | |
|---|---|---|---|---|---|
| | Total number of germs cfu/g | | | Removing level of NTG | |
| | Initial value | ANK 8mg /l chlorine | ANK 12mg /l chlorine | ANK 8mg /l chlorine | ANK 12mg /l chlorine |
| 1. Wheat | 50000 | 40000 | 3000 | 20.00% | 94.00% |

| | | | | | |
|---|---|---|---|---|---|
| 2. Maize | 30000 | 20000 | 10000 | 33.33% | 66.67% |
| 3. Maize gluten | 10000 | 1000 | 300 | 90.00% | 97.00% |
| 4. Rice | 300000 | 30000 | 20000 | 90.00% | 93.33% |
| 5. Barley | 60000 | 50000 | 30000 | 16.67% | 50.00% |
| 6. Soybean meal | 70000 | 60000 | 50000 | 14.29% | 28.57% |
| 7. Canola meal | 400000 | 300000 | 200000 | 25.00% | 50.00% |

Table nr. 3 shows the initial value of yeast and fungi for feedstuffs and after the treatment applied for 15 min with ANK solution containing 8 mg/l and 12 mg/l active chlorine.

Table nr.3- Removing level of yeast and fungi from feedstuffs with ANK solutions

| SAMPLE 10g feedstuff + 90ml ANK | Microbiological indicators | | | | |
|---|---|---|---|---|---|
| | Yeast and fungi cfu/g | | | Removing level | |
| | Initial value | ANK 8mg /l chlorine | ANK 12mg /l chlorine | ANK 8mg /l chlorine | ANK 12mg /l chlorine |
| 1 Wheat | 7000 | 6000 | 5000 | 14.29% | 28.57% |
| 2. Maize | 100000 | 10000 | 9000 | 90.00% | 91.00% |
| 3. Maize gluten | 1000 | 100 | 30 | 90.00% | 97.00% |
| 4. Rice | 10000 | 1000 | 900 | 90.00% | 91.00% |
| 5. Barley | 2000 | 200 | 180 | 90.00% | 91.00% |
| 6. Soybean meal | 3000 | 300 | 200 | 90.00% | 93.33% |
| 7. Canola meal | 300000 | 3000 | 2000 | 99.00% | 99.33% |

Table nr. 4 shows the initial value of NTG for diets and after the treatment applied for 15 min with ANK solution containing 8mg/l and 12mg/l active chlorine.

Table nr.4 – Removing level of NTG from diets with ANK solutions

| SAMPLE 10g diets + 90ml ANK | Microbiological indicators | | | | |
|---|---|---|---|---|---|
| | Total number of germs cfu/g | | | Removing level of NTG | |
| | Initial value | ANK 8mg /l chlorine | ANK 12mg /l chlorine | ANK 8mg /l chlorine | ANK 12mg /l chlorine |
| Lot Control – MDC | 200000 | 180000 | 140000 | 10.00% | 30.00% |
| Lot E1 – MDE1 | 250000 | 200000 | 170000 | 20.00% | 32.00% |
| Lot E3 – MDE3 | 140000 | 120000 | 100000 | 14.29% | 28.57% |

Table nr. 5 shows the initial value of yeast and fungi for diets and after the treatment applied for 15 min with ANK solution containing 8mg/l and 12mg/l active chlorine.

Table nr.5 - Removing level of yeasts and fungi from diets with ANK solutions

| SAMPLE 10g diets + 90ml ANK | Microbiological indicators | | | | |
|---|---|---|---|---|---|
| | Yeast and fungi cfu/g | | | Removing level | |
| | Initial value | ANK 8mg /l chlorine | ANK 12mg /l chlorine | ANK 8mg /l chlorine | ANK 12mg /l chlorine |
| Lot Control – MDC | 300000 | 270000 | 200000 | 10.00% | 33.33% |
| Lot E1 – MDE1 | 400000 | 300000 | 230000 | 25.00% | 42.50% |
| Lot E3 – MDE3 | 500000 | 400000 | 350000 | 20.00% | 30.00% |

*b) The eggshell analysis*

Table nr.6 shows the value of NTG for eggshells samples from laying hens fed on with tape water and ANK solutions respectively.

Table nr. 6 – Removing level of NTG on eggshell from laying hens fed on with ANK solutions

| SAMPLE<br>Eggshell | Microbiological indicators<br>Total number of germs cfu/g |
|---|---|
| Control lot control diet + tap water | 80000 |
| L1 E1 diet 1+ 8 mg/l active chlorine in water | 50000 |
| L1 E3 diet 3+ 8 mg/l active chlorine in water | 20000 |
| L2 E1 diet 1+ 12 mg/l active chlorine in water | 17000 |
| L2 E3 diet 3+ 12 mg/l active chlorine in water | 14000 |

Table nr.7 shows the initial value of NTG for eggs from control lot and after the treatment applied for 15 min with ANK solution containing 8mg/l and 12mg/l active chlorine.

Table nr.7- Removing level of NTG on eggshells from laying hens fed on with tap water, by immersion in ANK solutions for 15 min

| SAMPLE<br>Eggshell | Microbiological indicators | | | | |
|---|---|---|---|---|---|
| | Total number of germs cfu/g | | | Removing level | |
| | Initial value | ANK 8mg /l chlorine | ANK 12mg /l chlorine | ANK 8mg /l chlorine | ANK 12mg /l chlorine |
| Control lot | 80000 | 40000 | 30000 | 50.00% | 62.50% |

*c) The sanitation tests analysis*

The microbiologic analysis results for sanitation tests are showed in the Table nr.8.

Table nr.8 - Removing level of NTG and total coliform bacteria from hen-house space with ANK solutions

| SAMPLE<br>Floor | Microbiological indicators | |
|---|---|---|
| | Total number of germs/$cm^2$ | Total coliform bacteria / $cm^2$ |
| Sanitation test before washing | 1000 / $cm^2$ | + |
| Sanitation test after washing for 15 min with ANK, 36 mg/l active chlorine | 500 / $cm^2$ | - |

They were taken samples of feedstuffs, diets and eggs from INCDBNA Balotesti experimental house with electrolyzed water. For all samples were determined the initial microbial load. The initial value of NTG from feedstuffs varies in a large range (about ten times), between 10000 cfu/g for maize gluten and the highest value 400000 cfu/g, for canola meal while that of the mixed diets varies in a small range (about 1.8 times), between 140000 cfu/g for MDE3 and the highest value 250000 cfu/g, for MDE1.The initial value of NTG from eggshells presented an intermediate variation (about 5.7 times), between 14000 cfu/g for eggs from L2-E3 and the highest value 80000 cfu/g, for eggs from control lot.

The feedstuffs and diets samples were treated for 15 min with ANK solution containing 8mg/l respectively 12 mg/l active chlorine.

The ANK solutions containing 8mg/l active chlorine used in experiments had a bactericidal effect reducing the *NTG* content of feedstuffs from 14.29 % for soybean meal until 90.00 % for rice and maize gluten and the *yeasts and fungi* from 14.29 % for wheat until 99.00 % for canola meal. For mixed diets the reducing of NTG was from 10.00 % for Control-MDC until 20.00 % for MDE1 and the *yeasts and fungi* from 10.00 % for Control-MDC until 25.00 % for MDE1.

The ANK solutions containing 12mg/l active chlorine used in experiments had a better bactericidal effect reducing the NTG content of feedstuffs from 28.57% for soybean meal

until 97% for maize gluten and the *yeasts and fungi* from 28.57% for wheat until 99.33% for canola meal. For diets the reducing of NTG was from 28.57 % for MDE3 until 32.00% for MDE1 and the *yeasts and fungi* from 30.00 % for MDE3 until 42.50% for MDE1. Feedstuffs and diets with a higher microbial load need an ANK solution with high contents of active chlorine up to 15mg/l.

The NTG on eggshells from laying hens, who were fed on for least one month with ANK solution with 12 mg/l active chlorine were between 14000-17000 cfu/g. These values of NTG were significantly lower than 80000 cfu/g that was found for eggshells from laying hens fed on only with tap water. The NTG value is lower and lower with the increasing the active chlorine concentration in drinking water. When used in farm the content of active chlorine from drinking water has to be below 15mg/l. A higher content of active chlorine could affect the nutritional metabolism of laying hens. Both ANK solutions used in experiments had a bactericidal effect reducing the *NTG* content of eggshell with 50% for 8mg/l active chlorine respectively with 62.50% for 12 mg/l active chlorine.

Samples were taken from the floor before and after washing for 15 min with ANK solution containing 36 mg/l active chlorine. The reduction of NTG/cm$^2$ was for two times and the Coliforms were total removed. As technological method, ANK can be used as disinfecting agent by spraying for 15-30 min weekly, the housing area of laying hens. Cold fog of ANK could be applied with or without the laying hens inside. In the presence of hens, the concentration of active chlorine from ANK solution has not to be over 50mg/l to avoid breathing disorder. In order to have a maximum efficiency in the housing area without hens the ANK applied as disinfectant, should not be diluted.

## 4. Conclusion

We can conclude that the ANK solution supplied to laying hens in percentage of 1.5% - 3.0% in drinking water reduces the content of germs even inside the laying hens' body, diminishing the potential of eggs contamination. No significant differences were observed between the group which was given water at 12 mg/l respectively 8 mg/l active chlorine. Both ANK solution of 12 mg/l respectively 8 mg/l active chlorine had more effect for reducing NTG and *yeasts and fungi* from feedstuffs than that from mixed diets.

The quality of the administrated water and of mixed diets to the laying hens represents an important factor that influences laying hen health status and productive performances. ANK solution can be used efficiently to reduce the contents of NTG, *yeasts and fungi*, total coliform bacteria and other contaminants from drinking water, mixed diets and hen-house space, being considered a nontoxic product for poultry, as well, for the environment.

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