Holistic efficacy of specific nutrient synergy against avian flu virus: pathology and immunomodulation

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Summary

The authors evaluated the holistic efficacy of nine specific nutrient synergy (NS) against avian influenza virus (AIV) strain Lebanon 1 (H9N2). The study included two segments; the first was designed to determine the minimum dose among four doses (1X, 2X, 3X and 4X in which X = 24.4 mg/ml/bird) of NS, administered intraoesophageally, once per day between 7 and 14 days of age, resulting in an improvement of chicken performance without any toxic side-effects; the second aimed at reducing pathological effects and inducing immunomodulation by the determined safe dose of NS in chickens exposed to AIV. The first segment showed that the daily oral administration of the NS to birds between 7 to 14 days of age at the 2X dose-level (320 mg/kg body weight or 48.8 mg/ml/bird) resulted in a consistent and significant improvement in the feed conversion ($P<0.05$) at 10 and 14 days of age, associated with a significant ($P<0.05$) increase in the liver weight index. In addition, the administration of NS at 2X level resulted in complete absence of toxicity signs (swollen infraorbital sinuses, ocular exudate, nasal discharge, thick oral saliva, diarrhoea, lameness and huddling) and complete absence of toxicity lesions (airsacculitis, hydro-pericardium signs, pericarditis, perihepatitis, splenomegaly and tracheitis). The four groups of birds that received levels 1X to 4X levels had significantly higher frequency of birds with gaseous caeca compared to the control group deprived of NS ($P<0.05$), a sign of higher fermentation activity in this organ. Data from the second segment of this research showed that the daily administration of NS at a level of 48.8 mg/ml/bird, between 7 to 14 days of age, to H9N2-challenged birds reduced specific pathological effects at 14 days of age namely: absence of rales at 3 days post H9N2 challenge and gross lesions (absence of tracheitis and enteritis at 7 days post challenge). Such reductions in signs and gross lesions were associated with a 63.4% reduction in immune responses to the hemagglutinin protein of the AIV, an indication that NS has a reducing effect on the viral infectivity in chickens.

Keywords

Avian influenza, Chickens, Immunomodulation, Nutrient synergy, Viruses.

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Efficacia olistica di *nutrient synergy* specifiche contro il virus dell'influenza aviaria: patologia e immunomodulazione

Riassunto

Gli autori hanno valutato l'efficacia olistica di nove nutrient synergy *(NS) specifiche contro il virus dell'influenza aviaria (AIV). Lo studio ha incluso due fasi: la prima è stata messa a punto per determinare la dose minima su quattro (1X, 2X, 3X e 4X, dove X= 24,4 mg/ml/volatile) di NS, somministrata via intraesofagea una volta al giorno tra i 7 e i 14 giorni d'età, in grado di ottenere come risultato un miglioramento della performance produttiva dei polli senza effetti collaterali tossici; la seconda fase è stata rivolta, in polli esposti al virus dell'Influenza Aviare (IA), alla riduzione degli effetti patologici e all'induzione di immuno-modulazione mediante una dose riconosciuta come sicura di NS. La prima fase ha mostrato che la somministrazione orale giornaliera di NS in volatili di età compresa tra i 7 e i 14 giorni, ad una dose 2X (320 mg/kg per peso corporeo o 48,8 mg/ml/volatile) ha ottenuto come risultato un consistente e significativo miglioramento dell'indice di conversione dei mangimi (P<0,05) ad un'età compresa tra i 10 e i 14 giorni, associato ad un aumento significativo (P<0,05) nell'indice di peso epatico. Inoltre, la somministrazione di NS ad una dose 2X ha avuto come risultato una totale assenza di segni di tossicità (tumefazione dei seni infraorbitali, essudato oculare, scolo nasale, salivazione densa, diarrea, zoppia e affollamento) e di lesioni di tossicità (aerosacculite, segni d'idropericardio, pericardite, periepatite, splenomegalia e tracheite). I quattro gruppi di volatili che hanno ricevuto dosi da 1X a 4X hanno registrato una frequenza significativamente più elevata di animali con gas intestinale nei ciechi rispetto al gruppo di controllo non trattato con NS (P<0,05), segno di una maggiore attività di fermentazione intestinale. I dati della seconda fase di questa ricerca hanno mostrato che la somministrazione giornaliera di NS ad una dose di 48,8 mg/ml/volatile, tra i 7 e i 14 giorni d'età, in volatili infettati con H9N2 riduceva conseguenze patologiche specifiche a 14 giorni d'età, vale a dire assenza di rantoli nei 3 giorni dopo il challenge con H9N2, e di lesioni macroscopiche (assenza di tracheite ed enterite a 7 giorni dopo il challenge). Tali riduzioni di segni e di lesioni macroscopiche sono state associate ad una riduzione del 63,4% delle risposte immunitarie alla emoagglitinina dell'H9N2: un'indicazione che la NS ha l'effetto di ridurre l'infettività dell'H9N2 nei polli.*

Parole chiave

Influenza aviaria, Immunomodulazione, NS, Polli, Virus.

Introduction

Previous studies conducted by the authors have shown that a combination of nutrient synergy (NS) required for natural stability of the connective tissue in the body can effectively inhibit key mechanisms involved in invasion and growth of a large variety of cancer cells both *in vitro* and *in vivo* (18, 19, 20). These studies were based on the Rath concept that defines connective tissue stability as the main therapeutic target in carcinogenicity as well as in viral and bacterial infections (17). Consequently, the effectiveness of this approach in cancer triggered our interest in expanding an application of this NS to viral infections, such as the avian influenza virus (AIV).

The tested nutrient combination comprises nine nutrients essential for synthesis and stability of the connective tissue as well as for many other metabolic pathways in the body (18, 20). NS contains standardised green tea extract (23.6%), vitamin C (16.8%), L-lysine (23.6%), L-proline (17.7%), L-arginine (11.8%), N-acetyl cysteine (4.7%), copper (0.05%), manganese (0.02%) and selenium (trace).

The green tea extract contains the epigallo-catechin gallate (EGCG)-active ingredient that contains antioxidant and anti-carcinogenic properties (18, 20). EGCG also has an antiviral effect on influenza virus subtypes as previously documented (23), suggesting that the antiviral effect of EGCG on influenza virus is mediated by specific interaction with the hemagglutinin (HA) protein altering the physical properties of viral coat and by

suppressing viral RNA synthesis in Madin-Darby canine kidney (MDCK) cells.

Vitamin C is a strong antioxidant that plays an important role in stabilising the connective tissue by supporting collagen production and structure (12). Vitamin C is also known to have an antiviral effect on the influenza virus (7). It was found that mega-doses of vitamin C had an 85% prevention and relief of cold and flu symptoms in students between 18 to 30 years of age. Vitamin C, in combination with vitamin E, has a stronger effect on reduction of influenza virus infectivity, probably through the recycling effect of vitamin C on the tocopheryl compound of vitamin E (26).

The antioxidant properties of EGCG and vitamin C may play an important role in the anti-viral effect against influenza virus. It was demonstrated that oxidant-treated anti-protease is unable to prevent trypsin from cleaving the hemagglutinin protein (HA0) to HA1/HA2, resulting in a 10 000-fold increase in infectious influenza virus (9). As a protective effect, anti-proteases are present on the surface of alveoli and are inactivated by reactive oxygen species (ROS). Consequently, the use of antioxidant would be of primary importance to reactivate anti-protease to prevent influenza viral infections (16, 22).

Lysine, proline and arginine showed anti-carcinogenic effects against fibrosarcoma cells HT-1080, along with vitamin C and EGCG (20), HTLV-1 positive and negative leukaemia cells (8), human breast cancer lines MDA-MB-231 and MCF-7 (18), against human prostate cancer (10, 19) and many other cell lines. However, the literature available indicates that the antiviral effect of amino acid supplementation in diets is still meagre. The N-acetyl cysteine significantly decreased mortality in influenza-infected mice (27). A combination of N-acetyl cysteine with ribavirin had a synergistic effect in protection against lethal influenza viral infection in mice (6).

It has been reported that L-lysine could be an alternative therapy against genital herpes (2), while others observed that the addition of proline-rich polypeptide to resident peritoneal cells from female mice, infected with vesicular stomatitis virus (VSV), inhibits viral replication and results in a reduction of 4-logarithmic units in its titre (15).

The effect against influenza virus was also shown by elements present in NS, such as selenium and copper. Selenium deficiency increases the pathogenicity of influenza virus infection in mice (3). The increased oxidative stress due to influenza virus infection in selenium-deficient mice leads to an increased NF-κB expression, leading to an enhanced Th-2 cell response which results in an increased lung inflammation. On the other hand, mice with adequate selenium intake showed a Th-1 response after an influenza virus infection, leading to a lower pathogenicity effect. Copper manifested an anti influenza A effect through inhibition of the proton translocation machinery in M2 protein of this virus (5). Moreover, cloth dyed with a combination of gentiane-violet and copper showed significant antiviral activity against influenza A virus (13).

To our knowledge, no previous literature has documented the use of the nine specific nutrient synergy present in Epican Forte in reduction of pathological effects and immunomodulation in chickens infected with AIV.

The purpose of this study was to determine the appropriate dose of NS that improves the performance of chickens without any toxic side-effects and to study the effect of the determined dose on the reduction of pathological effects of the challenged AIV and immunomodulation to its H9-hemagglutinin protein.

Materials and methods

Segment 1: nutrient synergy-dosing experiment

Birds

Fifty meat-type day-old chicks were divided into five groups, each comprised 10 chicks. Group 1 served as controls and received no NS; while groups 2, 3, 4 and 5 received daily doses of 1X, 2X, 3X, 4X, respectively, between

7 and 14 days of age (equivalent to 24.4, 48.8, 73.2 and 97.6 mg/ml/bird, respectively).

Nutrient synergy administration

The NS product provided by the Rath Research Institute in Santa Clara, California, was dissolved in drinking water to yield the four concentrations mentioned above. Each bird received one millilitre of the assigned solution, intraoesophagally, once daily, at between 7 to 14 days of age. Each bird in control group 1 received 1 ml of drinking water, intraoesophagally, facing the same stress of catching and holding as that experienced in birds administered the NS. Feed and drinking water were administered *ad libitum* and according to 1984 nutrient requirements established by the National Research Council (NRC) (14).

Toxicity signs and mortality records

The following signs of toxicity were observed and recorded at 7 (just before the initiation of NS administration), 10 and 14 days of age namely: swollen infraorbital sinuses, ocular exudate, nasal discharge, thick oral saliva, diarrhoea, lameness and huddling. The daily mortality in each group was also recorded.

Gross lesions of toxicity

All birds in the five groups were sacrificed by CO_2 asphyxiation, at 14 days of age. The following gross lesions of toxicity were observed and recorded: airsacculitis, hydro-pericardium, pericarditis, perihepatitis, splenomegaly and tracheitis.

Body weights and feed conversion

The average body weight in each group was determined at 7, 10 and 14 days of age. The feed conversion ratio was determined in each group by dividing the feed consumed by live body weight. The presence of obvious gas bubbles in the caeca, reflecting higher feed component(s)-fermentation activity by the colonised normal microbiota of this organ, was recorded in each bird at 14 days of age.

Liver weight indices

The liver weight indices were determined in all groups at 14 days of age by dividing each liver weight by the respective live body weight ×100, thus allowing for the comparison of the average liver weight index among the five groups.

Statistics

The frequency of birds, among the five groups, that showed a specific sign at each age (7, 10 and 14 days), or a specific lesion at 14 days, was compared using the Chi-square test. The same statistical method was used to compare the frequency of presence of gaseous caeca in the different groups. The average body weight was compared among the five groups at each of the following three ages namely: 7, 10 and 14 days using the one way analysis of variance (ANOVA). In addition, feed conversion at 10 and 14 days of age and liver weight indices at 14 days of age were compared between the five groups by using the one way ANOVA. Cumulative mortality frequencies were compared among the five groups using the Chi-square test. Statistical computing, implementing the Chi-square and one way ANOVA, was performed using the Statistical Package for the Social Sciences (SPSS) program version 14.0 (SPSS Inc., Chicago).

Segment 2: pathology and immunomodulation in avian influenza-infected birds treated with nutrient synergy

Birds

Sixty meat-type day-old chicks were divided into three groups of 20 birds per group. Each group was reared in separate isolation rooms, with similar environmental conditions, the same availability of feeders and drinkers (feed and drinking water were available *ad libitum* and in accordance with the NRC nutrient requirements, (14).

Avian influenza challenge and nutrient synergy treatment

Birds in the three groups were reared similarly until the age of 6 days. Each bird in groups 2 and 3 received the same challenge of AIV mentioned below at 7 days of age. The virus used in the challenge was isolated in 2004 from a broiler chicken in a flock of 20 000 birds affected by avian influenza; the outbreak resulted in a total mortality of about 50%. The virus was subtyped as H9N2 at the American

University of Beirut and was confirmed by the Central Veterinary Laboratory in Weybridge by Ruth Manvel. The name of the strain is Lebanon AI 1, a virulent AIV. The number of passages done for the virus in 10-day chicken embryos is three, and the $TCID_{50}$ in 2 HA titre of the virus is 0.6×10^5/0.5 ml. Briefly, each bird received a volume of 0.5 ml of chick embryo allantoic fluid intratracheally, containing 2 HA units of the virus, equivalent to approximately 0.6×10^5 $TCID_{50}$/0.5 ml. Birds in group 1 were not challenged or treated (controls).

Birds in group 3 were the only birds that were administered NS intraoesophagally (1 ml/bird), on a daily basis, between the age of 7-14 days. Dosing of NS was based on the data obtained from the first segment of this work, namely, offering each bird in group 3 an amount of 48.8 mg/ml/bird/day.

Pathological signs and lesions

Pathological signs at 7, 10 and 14 days of age, and gross lesions in different organs at 14 days of age, were recorded for each bird in the three groups. The pathological signs observed included nasal discharge, ocular exudate, thick oral saliva, diarrhoea, lameness, huddling, swollen infraorbital sinuses and rales. The gross lesions observed in different organs included tracheitis, airsacculitis, splenomegaly, pericarditis, hydropericardium, enteritis, pancreatitis and perihepatitis.

Immunomodulation by nutrient synergy

Individual blood samples were collected from the jugular vein of 10 randomly selected day-old chicks. The blood collection was repeated at 7 and 14 days of age from the jugular vein of 10 randomly chosen birds of each of the three groups of chickens. Serum was collected from each blood sample and stored at –20°C. All stored sera were thawed at room temperature at the same time and each serum sample was subjected to the hemagglutination inhibition (HI) test (1).

Briefly, each serum sample was serially diluted in round-bottom wells of a microtitre plate, using a dilution factor of 1:2 and a saline diluent. The volume of the different dilutions of the serum sample per well was 50 µl. A volume of 50 µl of AIV, strain Lebanon 1, at 4 HA units was added to each serum dilution. The antibodies in the serum and the AIV antigen are allowed to react at room temperature for a period of 30 min. A volume of 50 µl of 1% chicken red blood cells (RBC) was added to each serum-antigen mix, stirred gently and kept for 45 min. The HI titre was read for each serum sample, which is the highest dilution of the serum sample that still has sufficient antibodies to bind to the hemagglutinin protein of the H9N2 virus, thus inhibiting this protein from binding to its receptor on the added chicken RBC, and resulting in RBC sedimentation at the bottom of the microtitre wells.

Statistics

Frequencies of birds (number of positive birds/total number of tested birds) that showed a specific sign or pathological effect were compared between the three groups using the Chi-square test. The mean HI titre was compared between the three groups using the one way ANOVA. Significant differences in the parameters measured were determined at P=0.05. The SPSS v.14 program was used for statistical computing of the data in segment 2 of this work.

Results and discussion

Segment 1: nutrient synergy-dosing experiment

All birds in the five groups showed an absence of symptoms at the beginning of the experiment (7 days of age) (Table I). The administration of NS in different doses (1X-4X) did not result in any signs of disease at 10 days of age (first 3 days of NS administration) (Table II) and at 14 days of age (after seven days of NS administration) (Table III).

There was an absence of gross lesions at the end of course of NS administration (Table IV), except for the gaseous caeca observation in all groups to which NS had been administered, compared to its absence in the untreated controls (Table V).

The gaseous caeca present in birds that had received NS indicate an enhanced feed component(s)-fermentation by the normal

Table I
Signs of disease at the commencement of Epican Forte administration in 7-day-old birds

Group[a]	Epican Forte dosage[b]	Number of birds with specific signs/Number tested						
		Swollen infraorbital sinuses	Ocular exudate	Nasal discharge	Thick saliva	Diarrhoea	Lameness	Huddling
1	–	0/10	0/10	0/10	0/10	0/10	0/10	0/10
2	1X	0/10	0/10	0/10	0/10	0/10	0/10	0/10
3	2X	0/10	0/10	0/10	0/10	0/10	0/10	0/10
4	3X	0/10	0/10	0/10	0/10	0/10	0/10	0/10
5	4X	0/10	0/10	0/10	0/10	0/10	0/10	0/10

a) group 1 comprises controls, deprived of Epican Forte; groups 2, 3, 4 and 5 received 1X, 2X, 3X and 4X doses of Epican Forte, effective day 7, respectively

b) 1X dose level equivalent to 24.4 mg/ml/bird (approximately 160 mg of Epican Forte/kg of body weight)

Table II
Number of birds that showed different signs depending on Epican Forte dosage at 10 days of age

Group[a]	Epican Forte dosage[b]	Number of birds with specific signs/Number tested						
		Swollen infraorbital sinuses	Ocular exudate	Nasal discharge	Thick saliva	Diarrhoea	Lameness	Huddling
1	–	0/10	0/10	0/10	0/10	0/10	0/10	0/10
2	1X	0/10	0/10	0/10	0/10	2/10	0/10	0/10
3	2X	0/10	0/10	0/10	0/10	0/10	0/10	0/10
4	3X	0/10	0/10	0/10	0/10	0/10	0/10	0/10
5	4X	0/10	0/10	0/10	0/10	0/10	0/10	0/10

a) group 1 comprises controls, deprived of Epican Forte; groups 2, 3, 4 and 5 received 1X, 2X, 3X and 4X doses of Epican Forte, effective day 7, respectively

b) 1X dose level equivalent to 24.4 mg/ml/bird (approximately 160 mg of Epican Forte/kg of body weight)

Table III
Number of birds that showed different signs depending on Epican Forte dosage at the end of the experiment (14 days of age)

Group[a]	Epican Forte dosage[b]	Number of birds with specific sign/Number tested						
		Swollen infraorbital sinuses	Ocular exudate	Nasal discharge	Thick saliva	Diarrhoea	Lameness	Huddling
1	–	0/10	0/10	0/10	0/10	0/10	0/10	0/10
2	1X	0/10	0/10	0/10	0/10	0/10	0/10	0/10
3	2X	0/10	0/10	0/10	0/10	0/10	0/10	0/10
4	3X	0/10	0/10	0/10	0/10	0/10	0/10	0/10
5	4X	0/10	0/10	0/10	0/10	0/10	0/10	0/10

a) group 1 comprises controls, deprived of Epican Forte; groups 2, 3, 4 and 5 received 1X, 2X, 3X and 4X doses of Epican Forte, effective day 7, respectively

b) 1X dose level equivalent to 24.4 mg/ml/bird (approximately 160 mg of Epican Forte/kg of body weight)

Table IV
Number of birds with different lesions depending on Epican Forte dosage at the end of the experiment (14 days of age)

Group[a]	Epican Forte dosage[b]	Number of birds with specific sign/Number tested					
		Tracheitis	Airsacculitis	Splenomegaly	Pericarditis	Perihepatitis	Hydro-pericardium condition
1	–	0/10	0/10	0/10	0/10	0/10	0/10
2	1X	0/10	0/10	0/10	0/10	2/10	0/10
3	2X	0/10	0/10	0/10	0/10	0/10	0/10
4	3X	0/10	0/10	0/10	0/10	0/10	0/10
5	4X	0/10	0/10	0/10	0/10	0/10	0/10

a) group 1 comprises controls, deprived of Epican Forte; groups 2, 3, 4 and 5 received 1X, 2X, 3X and 4X doses of Epican Forte, effective day 7, respectively

b) 1X dose level equivalent to 24.4 mg/ml/bird (approximately 160 mg of Epican Forte/kg of body weight)

Table V
Average body weight, feed conversion and number of birds with gaseous caeca in 14-day-old birds that received different doses of Epican Forte

Group[a]	Epican Forte dosage[b]	Average body weight[c] (g)	Feed conversion ratio[c]	Number of birds with gaseous caeca[d]
1	–	375.3[a]	2.24[a]	1/10[a]
2	1X	362.0[a]	1.67[b]	5/10[b]
3	2X	383.0[a]	1.69[b]	3/10[a], [b]
4	3X	357.0[a]	1.64[b]	7/10[b]
5	4X	374.4[a]	1.48[c]	7/9[b]

a) group 1 comprises controls, deprived of Epican Forte; groups 2, 3, 4 and 5 received 1X, 2X, 3X and 4X doses of Epican Forte, effective day 7, respectively

b) 1X dose level was equivalent to 24.4 mg/ml/bird (approximately 160 mg of Epican Forte/kg of body weight)

c) averages and feed conversion ratios with different superscripts are significantly different ($p<0.05$)

d) frequencies with different superscript letters are significantly different ($P<0.05$)

microbiota colonising the caeca, which is most likely to have been induced by the component(s) of NS. This observation was further reinforced by the data obtained in Tables V and VI where the feed conversion at 10 and 14 days of age had significantly improved in the birds to which NS had been administered, in comparison with that observed in the control birds deprived of NS ($P<0.05$). It is worth noting that the feed conversion ratio is defined as the amount of feed consumed over the body weight.

At the end of the experiment (14 days of age), groups receiving 1X, 2X and 3X doses of NS showed a similar feed conversion that was significantly better than the feed conversion of the controls ($P<0.05$) (Table VI). Furthermore, birds that had received the 4X dose had a feed conversion that was significantly better than the groups that had received doses 1X to 3X ($P<0.05$). This preliminary result of feed conversion is very important to the poultry industry, once extrapolated to millions of birds, which most likely will result in significant savings on feed consumption. It is worth noting that approximately 70% of the cost of rearing meat chicken is devoted to feed consumption (11).

The average liver weight index improved as the dosage of NS was increased from 1X to 4X (Table VII). The liver weight index of the four groups that received NS at different dosages

Table VI
Average body weight and feed conversion in 10-day-old birds that received different doses of Epican Forte

Group[a]	Epican Forte dosage[b]	Average body weight (g)[c]	Feed conversion ratio[c]
1	–	235.5[a]	2.63[a]
2	1X	224.0[a]	2.03[b]
3	2X	233.0[a]	2.16[b]
4	3X	241.5[a]	1.19[d]
5	4X	226.5[a]	1.45[c]

a) group 1 comprises controls, deprived of Epican Forte; groups 2, 3, 4 and 5 received 1X, 2X, 3X and 4X doses of Epican Forte, effective day 7, respectively
b) 1X dose level equivalent to 24.4 mg/ml/bird (approximately 160 mg of Epican Forte/kg of body weight).
c) averages and feed conversion ratios with different superscript letters are significantly different (P<0.05)

was statistically similar (P>0.05), but significantly higher than the liver weight index of the untreated control birds (P<0.05). This improvement in liver size of the NS-treated birds could have stimulated the anabolism processes in the liver, leading to a significant improvement of feed conversion to body weight.

Table VII
Average liver index in 14-day-old birds that received different doses of Epican Forte

Group[a]	Epican Forte dosage[b]	Average liver index[c]
1	–	3.9[a]
2	1X	4.3[a], [b]
3	2X	4.4[b]
4	3X	4.6[b]
5	4X	4.7[b]

a) group 1 comprises controls, deprived of Epican Forte; groups 2, 3, 4 and 5 received 1X, 2X, 3X and 4X doses of Epican Forte, effective day 7, respectively
b) 1X dose level was equivalent to 24.4 mg/ml/bird (approximately 160 mg of Epican Forte/kg of body weight)
c) liver index = liver weight/body weight ×100; averages with different superscript letters are significantly different (p<0.05)

Mortality was observed only in the group receiving the 4X dosages (10%) (Table VIII); while the other groups that had received NS (1X, 2X and 3X) and control birds had no mortalities.

The choice of the NS dosage to be used in AIV-infected poultry (segment 2 of this work) was based on results obtained in segment 1, as presented in Tables I-VIII. In spite of obtaining the best feed conversion in the group that receiving the 4X dosage, this level of NS administration would not be recommended in future experiments due to the level of 10% mortality recorded. Dosages 1X to 3X gave similar statistical feed conversions (P>0.05) (Table VI), associated with a moderate frequency of birds with gaseous caeca in the group receiving 2X doses (Table IV). This fact strengthens our future objectives to use 2X dosing to study the impact of NS in the reduction of pathological effects and immunomodulation in AIV-infected chickens.

Table VIII
Cumulative mortality in birds that received different doses of Epican Forte at the end of the experiment (14 days of age)

Group[a]	Epican Forte dosage[b]	Mortality rate[c]
1	–	0/10[a]
2	1X	0/10[a]
3	2X	0/10[a]
4	3X	0/10[a]
5	4X	1/10[a]

a) group 1 comprises controls, deprived of Epican Forte; groups 2, 3, 4 and 5 received 1X, 2X, 3X and 4X doses of Epican Forte, effective day 7, respectively
b) 1X dose level was equivalent to 24.4 mg/ml/bird (approximately 160 mg of Epican Forte/kg of body weight)
c) mortality rate followed by the same superscript letters are insignificantly different (P>0.05)

Segment 2: pathology and immunomodulation in avian influenza-infected birds treated with nutrient synergy

The signs of ailment were observed and recorded at different ages, namely, 7 days old (Table IX), 10 days old (Table X) and 14 days old (Table XI).

The eight signs of infection were absent from all birds in the three groups just prior to challenge at the seventh day of age (Table IX). This indicates that there was no disease outbreak among the birds during their rearing period between days 1 and 7. However, at 10 day of age (3 days after intratracheal challenge with AIV, strain Lebanon 1), only challenged group 2 that was deprived of NS showed a significantly higher frequency of birds with apparent rales, in comparison to challenged and NS-treated group 3 and to control unchallenged and untreated group 1 ($P<0.05$) (Table X). The other signs observed did not differ significantly between the three groups of birds at 10 days of age, namely: frequency of birds showing nasal discharge, ocular exudate, thick buccal saliva, diarrhoea, lameness, huddling and swollen infraorbital sinuses ($P>0.05$). This specific NS seems to have a significant protection effect on the respiratory system of birds against the controlled challenge by the AIV, strain Lebanon 1, with 100% prevention of rales at 3 days post challenge, which is the known incubation period of AIV (4). Moreover, at 14 days of age, the frequency of birds that exhibited signs of rales increased in both groups 1 and 2, in comparison to the frequencies at 10 days of age (Table XI). However, frequency was higher in group 2 deprived of NS in comparison to that in NS-treated group 3 ($P>0.05$). The other seven signs were almost nonexistent in birds from all three groups.

Table IX
Number of birds with different signs at the commencement of Epican Forte administration in 7-day-old birds

	Number of birds with specific signs/Number tested[b]							
Group[a]	Nasal discharge	Ocular exudate	Thick buccal saliva	Diarrhoea	Lameness	Huddling	Swollen infra-orbital sinuses	Rales
1	0/19[a]	0/19[a]	0/19[a]	0/19[a]	0/19[a]	0/19[a]	0/19[a]	0/19[a]
2	0/19[a]	0/19[a]	0/19[a]	0/19[a]	0/19[a]	0/19[a]	0/19[a]	0/19[a]
3	0/19[a]	0/19[a]	0/19[a]	0/19[a]	0/19[a]	0/19[a]	0/19[a]	0/19[a]

a) birds in group 1 were left without medication and were not challenged against avian influenza, group 2 was left without medication and challenged intratracheally with 0.5 ml of 2 HA units of avian influenza virus (AIV), group 3 was given Epican Forte from 7 to 14 days of age and challenged intratracheally with 0.5 ml of 2 HA units of AIV

b) figures with superscript letters are significantly different ($P<0.05$)

Table X
Number of birds showing different signs at 10 days of age

	Number of birds with specific signs/Number tested [b]							
Group[a]	Nasal discharge	Ocular exudate	Thick buccal saliva	Diarrhoea	Lameness	Huddling	Swollen infra-orbital sinuses	Rales
1	0/19[a]	0/19[a]	0/19[a]	0/19[a]	0/19[a]	0/19[a]	0/19[a]	0/19[a]
2	0/18[a]	0/18[a]	0/18[a]	0/18[a]	1/18[a]	1/18[a]	0/18[a]	11/18[b]
3	0/18[a]	0/18[a]	0/18[a]	0/18[a]	0/18[a]	0/18[a]	0/18[a]	0/18[a]

a) birds in group 1 were left without medication and were not challenged against avian influenza, group 2 was left without medication and challenged intratracheally with 0.5 ml of 2 HA units of avian influenza virus (AIV), group 3 was given Epican Forte from 7 to 14 days of age and challenged intratracheally with 0.5 ml of 2 HA units of AIV

b) figures with superscript letters are significantly different ($P<0.05$)

Table XI
Number of birds showing different signs at the end of the experiment (14 days of age)

Group[a]	Number of birds with specific signs/Number tested [b]							
	Nasal discharge	Ocular exudate	Thick saliva	Diarrhoea	Lameness	Huddling	Swollen infra-orbital sinuses	Rales
1	0/19[a]	0/19[a]	0/19[a]	0/19[a]	0/19[a]	0/19[a]	0/19[a]	0/19[a]
2	0/17[a]	0/17[a]	0/17[a]	0/17[a]	0/17[a]	0/17[a]	0/17[a]	16/17[b]
3	0/14[a]	0/14[a]	1/14[a]	0/14[a]	0/14[a]	0/14[a]	0/14[a]	12/14[b]

a) birds in group 1 were left without medication and were not challenged against avian influenza, group 2 was left without medication and challenged intratracheally with 0.5 ml of 2 HA units of avian influenza virus (AIV), group 3 was given Epican Forte from 7 to 14 days of age and challenged intratracheally with 0.5 ml of 2 HA units of AIV

b) figures with superscript letters are significantly different (P<0.05)

The gross lesions in different organs were recorded at 14 days of age (Table XII). Two gross lesions (tracheitis and enteritis) were absent in group 3 NS-treated birds, similar to the group 1 control birds (P>0.05) and significantly lower than the frequency of the two lesions present in NS-deprived birds of group 2 (P<0.05).

Such an observation indicates that NS probably hinders the replication of the challenged virus in the upper respiratory system of the trachea and in the intestine. It is worth noting that the H9N2 virus belongs to the group of AIV that multiply only in the respiratory and digestive system of chickens (24). This confinement of multiplication to the two systems is due to absence of a redundant basic amino acid sequence in the HA protein of the virus (21, 25) which prevents the protease protein in the host cells to break the HA protein into HA1 and HA2 components (24). The inability of the virus to transform its HA component to HA1 and HA2 by the host enzyme activity, prevents the H9N2 virus from causing a systemic viral infection (24). Documentation in the literature describing the pathogenic nature of H9N2 is in agreement with the observation of insignificant lesions in many visceral organs of the birds (spleen, heart, pancreas and liver) (Table XII) (the gross lesions of these visceral organs in challenged birds in groups 2 and 3 did not differ from those observed in the unchallenged control group 1) (P>0.05).

Immunomodulation

The specific immunomodulation by NS against the HA antigen of the AIV, strain Lebanon 1 is presented in Table XIII.

On day 1, the birds had an average maternal HI antibody against the HA protein of the H9N2 virus equivalent to 1:12.1, due to vaccination history of their parents (an inactivated vaccine against the virus, strain Lebanon 1). The maternal HI antibody titre of

Table XII
Number of birds showing different lesions at the end of experiment

Group[a]	Number of birds with specific lesions/Number tested[b]							
	Tracheitis	Airsacculitis	Spleno-megaly	Peri-carditis	Hydro-pericardium	Enteritis	Pancreatitis	Peri-hepatitis
1	1/19[a]	1/19[a]	0/19[a]	0/19[a]	0/19[a]	0/19[a]	0/19[a]	0/19[a]
2	8/17[b]	9/17[b]	0/17[a]	1/17[a]	0/17[a]	4/17[b]	0/17[a]	2/17[a]
3	0/14[a]	7/14[b]	0/14[a]	2/14[a]	0/14[a]	0/14[a]	0/14[a]	0/14[a]

a) birds in group 1 were left without medication and were not challenged against avian influenza, group 2 was left without medication and challenged intratracheally with 0.5 ml of 2 HA units of avian influenza virus (AIV), group 3 was given Epican Forte from 7 to 14 days of age and challenged intratracheally with 0.5 ml of 2 HA units of AIV

b) figures with superscript letters are significantly different (P<0.05)

Table XIII
Average hemagglutin inhibition titre specific to hemagglutinin of H9N2 avian influenza virus at 1, 7 and 14 days of age

Group[a]	Average hemagglutination-inhibition titre[b]	
	Day 7	Day 14
1	1:4.0[a]	1:1.5 [a]
2	1:4.7[a]	1:50.4[b]
3	1:3.6[a]	1:18.0[a], [b]

a) birds in group 1 were left without medication and were not challenged against avian influenza, group 2 was left without medication and challenged intratracheally with 0.5 ml of 2 HA units of avian influenza virus (AIV), group 3 was given Epican Forte from 7 to 14 days of age and challenged intratracheally with 0.5 ml of 2 HA units of AIV

b) figures with superscript letters are significantly different (P<0.05)

1:12.1 dropped at 7 days of age in the control group to 1:4, and at 14 days of age to 1:1.5, reflecting an absence of specific HI antibody against the challenged AIV in the experimental environment of the control birds. The rearing period that lasted 7 days ended with a similar drop of maternal antibody titre in the three groups (P>0.05). The challenge with the AIV at 7 days of age in groups 2 and 3 induced a clear infection titre seven days later, at 14 days of age, which was 2.8 times higher in the NS-deprived group 2 in comparison to the NS-treated group 3. This reflects that the NS treatment in group 3 was able to lower the infectivity of the AIV, thus dropping the immune system response by 64.3% in comparison to that of the NS-deprived birds in group 2 (23). This immunomodulation by NS corresponds to other segments of this research in which a significant lower rales frequency was noticed at 3 days post challenge in the NS-treated group 3 (Table X) and complete absence of tracheitis and enteritis gross lesions at 14 days of age (7 days post H9N2 challenge) in the same NS-treated group 3 (Table XII).

Conclusion

In conclusion, data from segment 1 of this research showed that daily oral administration of 48.8 mg/ml/bird NS to birds free of AIV infection and aged between 7 and 14 days, results in body weight improvement (P>0.05) and significant reduction in the feed conversion ratio (P<0.05) at 14 days of age, associated with a significant (P<0.05) increase in the liver weight index, a significant increase in caecal fermentation and complete absence of toxicity signs. The other data from segment 2 of this research indicate that NS treatment caused a significant reduction (P<0.05) of certain pathological effects namely: absence of rales at 3 days post H9N2 challenge and gross lesions (absence of tracheitis and enteritis at 7 days post challenge). This reduction in pathological effects was associated with apparent reduction in mean HI titre, which is most likely to be due to a reduction of the viral infectivity by NS.

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