Implementation of a pharmacologic protocol for testing bovine colostrum nutraceutical products in Broilers

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Abstract
As antibiotic additives were deemed illegal to use in poultry feed as a growth promoter but also to prevent certain diseases such as necrotic enteritis, researches are increasingly oriented towards finding an alternative that will provide both economic gain for the farmer and safety for the end consumer. Bovine colostrum, the first milk secretion postpartum, is widely known for its beneficial properties, not only on the newborn organism, but also in the adult one. The bioactive components of the colostrum have healing properties in the gut, furthermore they help prevent bacteria from adhering to the intestinal mucosa. The biochemical constituents also have an antibacterial effect through lactoferrin and lactoperoxidase. The main objective of this paper was to establish a protocol through which to obtain consistent scientific data when researching the effects of a nutraceutical product in poultry. In order to achieve this we have conducted a study, in the University of Agricultural sciences and Veterinary medicine, Cluj-Napoca, Romania, on a population of 60 broilers that were divided into three groups: the control group and two other groups that were administered distinct nutraceutical products in their drinking water. The purpose was to evaluate the effectiveness of replacing antibiotic growth promoters and anticoccidial drugs with nutraceutical products from bovine colostrum. Various microbiological, health and productivity parameters were assessed and compared between the groups, over a period of 45 days. The immunoglobulins from the bovine colostrum, as bioactive components, will achieve its highest peak in day 14 in the blood serum of the bird. As such, blood samples were deemed best to be harvested on EDTA and Clotting agents every 14 days, as the products were administered 2 times throughout the study. Cloacal swabbing was also performed, feces samples were evaluated for microbial concentration and bacterial strain identification. A comparison was made with several other researches that performed similar clinical studies and we recommend that when administering bovine colostrum nutraceuticals, in order to obtain scientifically consistent results, a strict protocol has to be implemented, periodical evaluations have to be made according to the parameters that are assessed but also in compliance with the bioactive components of the product.
Keywords: *Broiler, colostrum nutraceutical, microbial, parameter, protocol*

Introduction

In 1999 the American Association of veterinary medicine has described the nutraceutical products as micronutrients, macronutrients and other nutritional supplements used as therapeutic agents (Pandey et al., 2011). In 2019, a veterinary nutraceutical is described, by the North American Veterinary Nutraceutical Council, as being "a substance which is produced in a purified or extracted form and administered orally to patients to provide agents for normal body structure and function and administered with the intent of improving the health and well-being of animals."(Ramesh et al., 2019).

The poultry industry is particularly influenced by the use of nutraceuticals as the sub-therapeutic use of antibiotics in their feed has been either banned or reduced in many countries worldwide (Yesuf et al., 2017, Sugiharto, 2014), as it caused antibiotic-resistant microorganisms to develop and was a danger to consumers' health. Excluding antibiotics from the feed has caused

however numerous problems in the industry as the growth performance lowered and underlying diseases developed during the rearing period (Huyghebaert et al., 2011).

A wide range of nutraceuticals were used in the poultry industry in order to improve these aspects, from fenugreek, to ginger and neem extract and sea buckthorn (Yesuf et al., 2017, Mekuriya et al., 2018, Vlaicu et al., 2017, Ramesh et al., 2019), however the main objective of this paper was to establish a protocol through which to obtain consistent scientific data when researching the effects of a bovine colostrum nutraceutical product.

The bovine colostrum is well known for its bioactive and biochemical composition as their effects have been described since the 20th century (Parrish et al., 1950). The various growth factors have healing properties in the gut, moreover studies have shown that the same bioactive components improved muscle mass in athletes using colostrum nutraceuticals (Antonio et al., 2001). Another very important bioactive component are the antibodies. Researches show that over 60% of the total proteins in the bovine colostrum is represented by immunoglobulins and 90% of these are immunoglobulins G (IgG) which are specifically responsible for binding antigens, neutralizing toxins (Alexieva, 2004).

The biochemical components with antimicrobial properties in the bovine colostrum, lactoperoxidase and lactoferrin, are proven to be effective against Escherichia Coli and Salmonella typhimurium (Freedman et al., 1998, Xu et al., 1996). Furthermore, the lysozyme is a well-known enzyme with antibacterial effect on gram-positive and gram-negative microorganisms by destroying the bacterial wall (Reiter, 1978).

The lack of reliable guidelines and results when administering nutraceuticals in poultry has reduced the trust that veterinarians put in alternative medicine. Also, some statistics claim that the results of such clinical trials are published in less publicized scientific journals (Taillon et al., 2000).

Materials and methods

This study was conducted with the approval of the bioethics committee from the University of Agricultural Sciences and Veterinary Medicine, Cluj-Napoca, Romania, where the protocol was also implemented.

The birds, obtained from a local commercial hatchery, were from the Ross308 Broiler breed. The chicks (n=60) were 1 day old since hatching and clinically healthy. They were then raised in 3 rearing pens, 20 birds in each pen, randomly selected (T1, T2 and T3), in a controlled temperature room. Male and female chickens were reared together. The provided bedding consisted of natural, untreated wood shavings; all pens were equipped with an infrared light, of adequate intensity, feeders and water troughs. Thorough cleaning was performed every week when the bedding was also replaced. All birds were given commercial starter crumbled pellets at discretion during the first 17 days of life, after which the feed was changed to small crumbles until the last day of the clinical test. Moreover, during the first 4 days of life the feed was also provided on sheets of paper placed on the bedding in order to stimulate the food intake. The water was provided at a temperature of 20℃ and was changed 3 times a day during the first 3 weeks and 2 times a day during the last 2 weeks of the study. The temperature on the ground was maintained at 32-35℃ during the first week of life, after which it was reduced by 5 degrees with each week.

The testing followed a unicentric, randomized, cross-over protocol with two treatments and a pause between them (Fig. 1). The used protocol was adapted to evaluate the immune stimulation of two nutraceutical products obtained from bovine colostrum: one of them containing a set of bioactive immunoglobulins, lactoferrin, lactoperoxidase and lysozyme (P1), while the other product though also based on bovine colostrum whey, it was devoid of proteins, with added

the added strain NCIMB 11974 of Lactobacillus plantarum (P2). The target species, according to the producer, were cattle, horses, sheep, goats, swine, canines and felines for P1 and all species of mammals, birds and fish for P2.

The protocol commenced with organizing the study (day 0), administering the products (day 1- 3), harvesting the first 15 serial blood and feces samples, 5 from each group (day 17). It is worth mentioning the fact that in accordance to the producer, the products were to be administered during the first 3 days of life and during stress phases. Moreover, as P1 did not have birds as a target species, the dose in which it was administered was the one proposed for P2: 1ml of product in 1l of drinking water. Group T1 was offered clean water with nothing added while T2 was offered water with P1 and T3 water containing P2. All chicks were kept under the same managerial, hygienic and environmental conditions, had ad libitum access to feed and water throughout and were maintained on a constant 24 hours light schedule. Also, the water was maintained at a pH of 4.4 for the duration of the study. It is important to state the fact that the chicks were vaccinated on the first day of life against the Newcastle disease and, for experimental purposes, it was decided against any other vaccination for the duration of the clinical testing.

Procedure	**Phase 1**											
Study day	0	1	2	3	4	5	6	7	9	11	14	17
Physical examination	●											
Live weighing	●							●			●	
Vital signs	●	●										
Clinical chemistry												●
Haematology												●
Microbiology												●
Serology												●
Product administration		●	●	●								●
Blood collection for drug concentration												●
Adverse events observing and recording		●	●	●	●	●	●	●	●	●	●	●
	Phase 2											
Study day	18	19	20	21	22	23	28	31	32	33	35	45
Physical examination												●
Live weighing				●			●				●	
Vital signs												●
Clinical chemistry								●				
Haematology								●				
Microbiology								●				
Serology								●				
Product administration	●	●										
Blood collection for drug concentration								●				●
Adverse events observing and recording	●	●	●	●	●	●	●	●	●	●	●	●

Fig. 1. – The schematic representation of the implemented protocol in testing two colostral nutraceutical products in Broilers

The birds were weighed every week and the mortality and morbidity were recorded if any. At the end of the clinical trial, economic calculations were made based on the feed costs and feed consumed, the cost of the administered colostrum for each group and based on the gained weight.

As blood harvesting is a well-known stress factors for birds, day 17 was also chosen for the second treatment. As such during days 17-19 the products were again administered. And on day 31, another series of 15 blood and feces samples were harvested. The final examinations were performed at the end of the second phase, including repeating the clinical and para clinical examinations (day 45).

The individual hematological samples were harvested thus: 2ml of blood on EDTA and 1ml on Heparin. In order to achieve this, we have resorted to the puncture of the brachial veins, with 24G needles which were suitable for the caliber of not only the veins but also the size of the bird erythrocytes. Each sample was clearly identified with a number, the test group, date at which it was obtained. The feces samples were also individual, they were obtained through cloacal swabbing and deposited in sterile containers until processing.

The processing and analysis of the blood samples included two sets of investigations. The first consisted of the analysis of hematological indices: blood cell count, smears, hematocrit and hemoglobin. All the samples were immediately processed without refrigeration. Once the serum and plasmas were obtained, they were however stored at -20°C for processing at a later date for the second set of investigations. The plasma was obtained by centrifuging the blood samples at 2500 rpm for 5 minutes.

Once the feces samples were collected, they were immediately processed in order to assess the total bacterial concentration and for identification of gram-negative and gram-positive strains. Furthermore, on day 17, an average sample was collected from each group so as to identify parasitic infections.

Results and discussions

According to the Committee for Medicinal Products for Veterinary use (Committee for Medicinal Products for Veterinary use, 2012) this study is considered to be an exploratory one as it has clear objectives, with no specific hypothesis, allowing data exploration during the analysis, contributing to the proof of concept yet needing more research in order to establish its efficacy. As such, though not necessarily subjected to the Good Clinical practices, it is mandatory to be pre-planned and ethical. Several studies that also researched the effectiveness of nutraceutical products in poultry integrated this step into the design of the protocol (Fatih et al., 2018, Vlaicu et al., 2017, King et al., 2005).

Even though some studies have chosen to perform the trials on either male or female chicks (Gaucher, 2015, Torok et al., 2008), randomizing the individuals is recommended and practiced by many other researchers (Fatih et al., 2018, King et al., 2005, Campbell et al., 2004). The Guidelines on statistical principles for clinical trials for veterinary medicinal products clearly state that randomization "help(s) to avoid possible bias in the selection and allocation of subjects arising from the predictability of treatment assignment".

According to Dr. Jacquie Jacob from the University of Kentucky, wood shavings is the best bedding for poultry is wooden shavings as it is nontoxic to the birds, very absorbent, has reduced thermal conductivity(Jacquie, 2015). The only downside of this bedding is the economical side as for some countries it has become expensive due to high demand. In this regard, not all researches done on Broiler chickens took this aspect into consideration (Quereshi et al., 2004, Oe et al., 1975).

Acidifying the water of birds is a well-known procedure in order to reduce the prevalence of necrotic enteritis and coccidiosis (Ayhan et al., 2019, Sugiharto, 2014, Gaucher, 2015). The feed that was provided to the chicks was mainly composed of maize and soy-beans which was formulated to meet or exceed the standards for major nutrients for broiler starters (King et al., 2005). As other researches, our feed was pressed into cold-pellets at 60°C and it was pre-conditioned at with steam addition at the temperature of 60-70°C.

The temperature maintained on the ground, along with the light intensity provided by neon lights and the infrared lights were in accordance with the Ross308 Performance Objectives. As most researchers, the light program was for 24h and the heating was maintained at 32-35℃ during the first week of life, after which it was reduced by 5 degrees with each week (Gheorghe, 2013, Insha et al., 2018).

The duration of this study was 45 days, which is the average lifespan of a Broiler chicken according to the National Chicken Council in the United States, in 2019. Similar clinical tests also conducted their research during the same amount of time (Fatih et al., 2018, Yesuf et al., 2017, Vlaicu et al., 2017). As far as the number of treatments that were conducted, each study is unique and individual, however in the current case given that fact that P1 is based on the absorption of antibodies in the bloodstream, literature shows that the highest titer of antibodies is reached around day 14 after administration (Quereshi et al., 2004). The standard brachial vein harvesting is mentioned in numerous studies, and as far as the storage is concerned, it differed depending on the upcoming tests (Kalia et al., 2018, Gheorghe, 2013). As in our case, the serum and/or plasma were kept at temperatures of -20°C until processing (Ghasemi, 2006). As far as P2 was concerned, the gut microflora can be collected at any date after the administration for of product. According to other studies which focused on this particular aspect, collected the samples around day 4, 21, 35 and in most situations, the samples were pooled into an average sample (Maciuca et al., 2015). In our case, given the limited amount of individuals it was possible to obtain both and individual and a pooled sample.

A clinical trial is not relevant unless a control group is set. As such, our research was comprised of 3 groups, T1, T2 and T3, where T1 was the control group and did not receive any additives in their diet. All the studies taken into consideration also had this group well represented in the protocol (Yesuf et al., 2017, Quereshi et al., 2004).

For the duration of this study we chose to not vaccinate the broilers unlike the standards for poultry (Yesuf et al., 2017, Gaucher, 2015). We believe that this will bring a higher value to the results obtained during this trial. It is important to mention at this point that we had no mortality for the duration of the study, with no significant morbidity to report that was relevant to the results of the study. Weighing the chicks is an important step in a protocol for animals raised for economical purposes and in our study, we performed the live weighing every week (Fatih et al., 2018, Yesuf et al., 2017). Again, this step is different among other studies where this process was done every other week.

Conclusions

We consider that, in order to obtain relevant and consistent scientific information on which the medical society can rely, protocols for testing of nutraceuticals on experimental animals or target species should be developed and even standardized. This desideratum is also essential for poultry breeding because it can significantly contribute to limiting the use of medicated feed (with sub-dosage antibiotics) for preventive purposes or as growth promoters. We also appreciate that it is very important to prove the efficacy and safety of nutraceutical products, including those from bovine colostrum, before they are placed on the market. Given that this study has conducted

extensive and up-to-date documentation on the implementation of clinical trials on poultry, we consider that this protocol is in line with current conduct and the data obtained is relevant and highly applicable.

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