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CURRENTLY UNKNOWN ASPECTS OF POULTRY NECROTIC ENTERITIS PATHOGENESIS

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Abstract

Necrotic enteritis (NE) or poultry clostridiosis is a disease which poses enormous health problems and makes tremendous economic losses to intensive poultry production worldwide. Despite having been targeted in extensive research for decades, a number of aspects of its pathogenesis remain unknown. For more than 30 years alfa-toxin has been considered to be the main virulence factor of the causative agent, but experimental research using a mutant *Clostridium perfringens* strain lacking the gene coding for this confirmed that alpha-toxin is not necessary for pathogenesis. Since the 1980s, NetB toxin has been the main suspected virulence factor. However, recently it has been discovered that the large clostridial cytotoxin named TpeL also contributes to the pathogenesis of NE. In spite of that, the prevalence of the genes which code for these toxins vary between the isolates of *C. perfringens* from the intestines of diseased poultry, which made clear that further investigation into their roles is necessary. It has been agreed that specific intestinal environmental conditions, which favour the growth and multiplication of *C. perfringens*, are key factors to the emergence of disease. Given that a battery of non-specific factors contributes to pathogenesis, as well as that it is impossible to eliminate them in intensive poultry production, not much hope remains that NE can be controlled. In this short review, the current knowledge on the pathogenesis of NE has been summarized.

Key words: *Clostridium perfringens*, necrotic enteritis, poultry, NetB, TpeL

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I DANAS NEPOZNATI ASPEKTI PATOGENEZE NEKROTIČNOG ENTERITISA ŽIVINE

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Kratak sadržaj

Nekrotični enteritis (NE) ili klostridioza živine, predstavlja veliki zdravstveni problem i nanosi ogromne ekonomske gubitke intenzivnoj živinarskoj proizvodnji širom sveta. Brojni aspekti patogeneze NE su i danas nepoznati, uprkos tome što su decenijama predmet intenzivnih izučavanja. Više od 30 godina je alfa-toksin razmatran kao glavni faktor virulencije uzročnika, ali su eksperimentalna istraživanja primenom mutantnog soja *Clostridium perfringens*, koji nema gen koji kodira sintezu alfa-toksina, potvrdila da ovaj toksin nije neophodan za nastanak bolesti. Od osamdesetih godina prošlog veka, NetB toksin je "glavni osumnjičeni" faktor virulencije, a od nedavno se smatra da i veliki klostridijalni ekstracelularni citotoksin, koji je nazvan TpeL, doprinosi patogenezi NE. Međutim, prevalencija gena koji kodiraju ove toksine, veoma varira kod izolata *C. perfringens* iz creva obolele živine i jasno je da su neophodna dodatna ispitivanja njihove uloge. Saglasnost postoji da su za pojavu bolesti ključni specifični uslovi u crevima koji pogoduju rastu i umnožavanju *C. perfringens*. Ako uzmemo u obzir da niz nespecifičnih faktora tome doprinosi, kao i da ih je praktično nemoguće u potpunosti otkloniti u intenzivnoj živinarskoj proizvodnji, ne ostaje mnogo nade da je nekrotični enteritis moguće staviti pod kontrolu. U radu ukratko sumiramo trenutna stanovišta o patogenezi NE živine.

Ključne reči: *Clostridium perfringens*, nekrotični enteritis, živina, NetB, TpeL

INTRODUCTION

Poultry clostridiosis, also known as necrotic enteritis (NE), was first reported in Australia (Bennetts, 1930), but it took three decades to be described in detail in the United Kingdom (Parish, 1961). However, it was not listed in a British guide to common poultry diseases in 1964, nor in a review article on

global poultry production and health problems in 1979. What is more, even in 1997, it still failed to attract much attention in an international directory of poultry diseases. It was not until the beginning of the 21st century that NE started to be considered a global issue (Williams, 2005). A possible reason for the emergence of this problem is a continuous administration of antibiotics as growth promotors, for example virginiamycin and bacitracin, as well as anticoccidials, especially those of the ionophorous group. Besides being coccidiostats, the latter is known for their Gram-positive antibacterial activity which inhibits *C. perfringens* growth in the guts and these contributes to its antimicrobial resistance. The use of antibiotics other than those with coccidiostatic and histomonostatic effects added to broiler feed has been forbidden in the EU since 2006 (Regulation 1831/ 2003). A sharp rise in incidence of NE has resulted from the ban on growth-promoting antibiotics and the withdrawal of ionophore coccidiostats owing to vaccination against coccidia (Coper and Songer, 2009). Today NE makes huge losses to intensive poultry production worldwide, which are estimated to may reach about 2 billion US dollars per annum (Martin and Smyth, 2009; Coper and Songer, 2009). The disease mainly occurs in broiler chickens 2-6 weeks after hatching, or more precisely in those 4-weak-old, although cases have been described in older birds, even up to 6 months (Williams, 2005; Paiva and McElroy, 2014). In peracute and acute cases, mortality is high, sometimes reaching 50%, often without premonitory signs (Timbermont et al., 2011). Nowadays, for reasons not clear enough, subclinical diseases prevail (Van Immerseel et al., 2008). The losses result from reduced growth, increased feed conversion and carcass condemnation at slaughter (Bailey et al., 2013). Typical pathology is detected in the jejunum and ileum, which varies from multifocal ulcerations in less severe cases to greenish or yellowish pseudomembranes if extensive mucosal inflammation and necrosis develop (Ficken and Wages, 1997). Enabling bacteria, including *C. perfringens,* to enter the portal blood circulation, the intestinal damage may lead to cholangiohepatitis. Such livers are enlarged, pale, yellowish, with haemorrhages and white necrotic foci. These changes are mainly detected in abbatoirs, without signs in the flock which would suggest the pending outcome.

In spite of extensive research in the last several decades, NE still remains a disease with some aspects not entirely clear, or even unknown. This specially refers to virulence factors of the bacterium, which are of utmost importance to the pathogenesis.

THE CAUSATIVE AGENT AND SOURCES OF INFECTION

The causative agent of NE is *Clostridium perfringens*, an anaerobic spore-forming bacterium, widely distributed in nature: it can be detected in soil, dust, sewage, and fresh and marine water. Besides, it is a common inhabitant of the intestinal tract in both humans and other homeotherms (Songer, 1996; Brynestad and Granum, 2002). Depending on the production of four mayor toxins (alpha, beta, epsilon and iota) *C. perfringens* strains are classified in one of the five toxin types – from A to E (Hateway, 1990; Petit et al., 1999). These bacteria may produce at least 13 other toxic or potentially toxic exoproteins, although not one of them may produce all (Coper and Songer, 2009; Chen and McClane, 2015). As a rule, the causative agent of NE is of type A and rarely C (Songer et al., 1996).

Poultry continually ingest *C. perfringens* spores. They are found in poultry housing, as are isolated from the walls, floors, fans, from the water pipes, nipple-drinkers and drip-cups even before the chicks enter (Williams et al., 2005). In addition, poultry feed is frequently contaminated with *C. perfringens* spores, either from raw ingredients, or when processed, stored or distributed. For example, in Brazil, out of 80 raw samples of fresh feed ingredients (meat and bone meal, blood and feather meal, poultry viscera meal and vegetable mix samples), 60% were found to be positive for *C. perfringens* (Casagrande et al., 2013). Thus, feed contamination depends on its composition, the hygiene during production and the storage conditions, and is directly proportional to the levels of soil and faecal contamination (Wojdat et al., 2006; Milanov et al., 2018). Thermal treatments, the pellet and extrusion processes, typically involve exposure to about 85°C for only a few minutes, which is not high enough to kill *C. perfringens* spores (Williams, 2005). However, despite the lack of absolutely effective treatment to prevent contamination with *C. perfringens* spores, poultry feed is not necessarily contaminated while being produced. As *C. perfringens* is ubiquitous in farm environment, even if non-contaminated feed is brought in, 2 weeks after the introduction of chicks, spores are detectable in feed samples (Williams, 2005). However, the intake of *C. perfringens* spores will not inevitably lead to disease.

C. perfringens occurs naturally in healthy chickens' guts, not unlike some other bacteria, for example *Lactobacillus* and *Streptococcus* (Knarreborg et al., 2002). It is known that *C. perfringens* may invade the chick's intestines soon after hatching (Paiva and McElroy, 2014) and can be isolated from eggshell fragments, chicken fluff, and paper pads in commercial broiler hatcheries (Craven et al., 2001). Moreover, even newly hatched chicks may be carriers due to verti-

cal transmission of the bacteria (Shane et al., 1984). Insufficiently established gut microbiome, which plays an important role in natural defence against enteric pathogens and the immunity as a whole, hugely contributes to the susceptibility of young birds to NE. Intestinal microbiota decrease the susceptibility to pathogenic bacteria by competing for attachment sites, producing volatile fatty acids and thus shifting the pH to lower values. It has been proven that germ-free chickens were more sensitive to *C. perfringens* or its toxin in comparison not only to those with normal microbiota, but also to those invaded exclusively by *Lactobacillus acidophilus* or *Streptococcus faecalis* (Fukata et al., 1991). It is perfectly possible that changes in the guts, for example reduction in major intestinal microflora, lead to higher numbers of *C perfringens*, and consequently higher production of alpha-toxin which eventually results in the outbreak of NE (Fukata et al., 1991).

PREDISPOSING FACTORS

The presence of *C. perfringens* alone does not necessarily lead to disease development (Paiva and McElroy, 2014): firstly, its growth in the guts is naturally limited, and secondly, not every strain is capable of producing a disease. The limitation of *C. perfringens* growth in the intestines, which is an environment with limited amino acid available, is due to its incapacity to synthesize enzymes necessary for the production of 13 amino acids (Brynestad and Granum, 2002; Cooper and Songer, 2009; Antonissen et al., 2014). Thus, it is considered that the key factor in the emergence of NE is the intestinal environment which favours the growth of *C. perfringens* and the consequential production of extracellular toxins which will do damage to the intestines (Keyburn et al., 2006; Cooper and Songer, 2009). This can be supported by a number of factors: dietary factors, immune status, stress and comorbidity, and mycotoxins.

Dietary factors: Diets with high levels of indigestible, water-soluble non-starch polysaccharides (wheat, rye, oats and barley), protein-rich diets containing relatively high concentrations of poorly digestible proteins result in high protein concentrations in the gastrointestinal tract and thus act as substrates for the bacteria (Jia et al., 2009; Williams, 2005). High percentages of animal protein sources (fishmeal, meat and bone meal) (Drew et al., 2004) and animal fat (Knarreborg et al., 2002) in a diet, as well as decreased intestinal motility (Coper and Songer, 2009) can increase the odds of NE development. High protein content contributes to the increase in pH, which favours the growth and reproduction of *C. perfringens* (Williams, 2005). In chickens artificially infected with *C. perfringens* it was proven that the intake of feed contaminated

with deoxynivalenol (DON), a major mycotoxin, in concentrations of 3,000 to 4,000 μg/kg feed led to more than two-fold increase in the proportion of birds with subclinical NE in comparison with the control (Antonissen et al., 2014). The results undoubtedly showed that DON disrupts intestinal barrier and produces intestinal epithelial damage, which leads to an increased permeability of the gut wall and decreased absorption of dietary proteins, whose concentrations were significantly increased in the duodenal content. What is more, DON did not affect the *in vitro* growth, production of alpha-toxin and netB toxin transcription in *C. perfringens*. In other words, it influenced the gut wall architecture, but not the bacterium pathogenic potential. The resulting leakage of plasma proteins into the gut leads to higher protein concentrations in the intestinal lumen, and favours the growth of *C. perfringens*. In was concluded that feed contaminated with DON at concentrations even below the EU maximum acceptable level (5.0 mg/kg of complementary or complete feeding stuffs), is a predisposing factor for NE in broiler chickens (Antonissen et al., 2014).

Immune status, stress and comorbidity: Various stressors can also contribute to the development of NE, such as alterations in the feeding regime (replacing starter diets with grower diets, for example), or increase in stocking density. Comorbidity which lead to immunosuppression (simultaneous infection with some viruses: chick anaemia virus, Gumboro disease or Marek's disease viruses) is also detrimental to the integrity of the gut mucosa. The most well-known predisposing factor for NE is mucosal damage caused by various coccidia species, especially *Eimeria maxima*, due to their intracellular multiplication (Baba et al., 1997; Williams, 2005). It was suggested that even infection with roundworms, namely with Ascaridia species, may contribute to NE due to the intestinal wall damage caused by their larval development (Palliyeguru et al., 2014)

The role of predisposing factors is of utmost importance since in unfavourable intestinal conditions, even highly virulent *C. perfringens* strains fail to produce a disease (Coper and Songer, 2009; Paiva and McElroy, 2014). In healthy chicks' gut content the number of *C. perfringens* usually is up to 10^2 or 10^3 CFU/g (Baba et al., 1997), whilst in the intestines of birds suffering from NE the corresponding numbers may increase up to 10^6 or even 10^8 CFU/g (Cooper and Songer, 2009). However, even extremely high numbers of *C. perfringens* in gut contents do not inevitably cause NE (Coper and Songer, 2009), since not all strains are able to do so – only clostridia which possess host-specific virulence factors are pathogenic for poultry (Timbermont et al., 2011). Now it is known for sure that firstly, only certain strains are capable of inducing NE, secondly, it happens if some predisposing factors are present, and thirdly, these make up only a minority in the guts of healthy chickens (Timbermont et

al., 2011). Pulse-field gel electrophoresis (PFGE) or amplified fragment length polymorphism (AFLP) detected various genotypes of *C. perfringens* type A in healthy flocks, even within an individual and within the same part of intestine. PFGE proved a high degree of genetic diversity in isolates from healthy birds but little in those suffering from clinical NE (Cooper and Songer, 2009; Timbermont et al., 2009). In naturally infected diseased chicks, a single clone of *C. perfringens* is dominant in the intestines (Timbermont et al., 2011). Isolates of *C. perfringens* from a flock with NE generally belong to the same genotype, even if obtained from different animals or organs (Engström et al., 2003; Nauerby et al., 2003; Gholamiandehkordi et al., 2006). By contrast, Johansson et al. (2010) assessed the genetic diversity of 88 *C. perfringens* isolates from a single broiler flock affected by mild NE in Sweden and detected 32 genotypes with PFGE. In the majority of affected birds more than one genotype was identified, which was explained with the presumption that in mild NE not one virulent strain can achieve complete dominance as it is possible in acute diseases.

CLOSTRIDIUM PERFRINGENS VIRULENCE FACTORS

Despite the fact that NE has been known for such a long time, *C. perfringens* virulence factors which are of profound significance to the pathogenesis are yet to be identified. For more than 20 years alpha (a) toxin, a zinc-dependent phospholipase/sphingomyelinase C, was considered to be the main factor of virulence in the pathogenesis of NE (Songer, 1996; Van Immerseel et al., 2008), although its precise role in disease development has not been understood fully (Keyburn et al., 2006). Al-Sheikhly and Truscott (1977), for example, successfully reproduced NE in birds infused intraduodenally with bacteria-free crude toxin. In this research it was revealed that alpha-toxin is the most dominant protein present in crude supernatants but the others were neglected, although they could possibly add to its effects or even lead to NE. Earlier studies which confirmed the role of alpha-toxin in chicks inoculated with the supernatant of *C. perfringens* cultures have a certain drawback: they did not take into account the possible presence of some other toxins. The most solid evidence that alpha-toxin is not the key virulence factor is the experimentally induced NE with a mutant lacking alpha-toxin (Keyburn et al., 2006). The role of alpha-toxin in the pathogenesis of NE was doubted also in the research conducted by Thompson et al. (2006). All toxotypes of *C. perfringensa* produce alpha-toxin, but only some of them, types A and C, can cause NE in chickens. It is clear that, if alpha-toxin were sufficient to cause the disease, all *C. perfringens* types would be the causative agents of NE, which obviously is not true.

Some further research has also suggested that alpha-toxin plays only a minor role in the pathogenesis of NE disease. NE in chicks is characterised by granulocyte migration to the intestinal lumen (Olkowski et al., 2006), which is a reaction very different from the leukostasis and lack of inflammatory response induced by alpha-toxin in gas gangrene. These facts additionally support the theory that NE lesions are mediated by some other toxins rather than alpha-toxin. *In vivo*, the substrates for alpha-toxin are phosphatidylcholine and sphingomyelin, which are both components of biological membranes, and so of the guts' epithelial cells. However, the tissue lesions in the early phases of NE do not correspond to the activities of phospholipase and sphingomyelinase of the alpha-toxin (Olkowski et al., 2008).

Keyburn et al. (2008) used a gene knockout mutant and discovered a novel pore-forming toxin – NetB - in a *C. perfringens* strain isolated from chickens with NE in Australia, claiming that NetB is a major virulence factor associated with this chicken disease. Regarding the amino acid sequences NetB toxin is partially similar to some other pore-forming toxins, for example to beta toxin of *C. perfringens* (38% identity) and alpha-toxin produced by *Staphylococcus aureus* (31% identity). NetB forms pores of at least 1.6 nm in diameter in cellular membranes, causing an influx of ions (Ca, Na, Cl etc.) which eventually leads to osmotic cell lysis (Savva et al., 2013). Mature NetB toxin is of similar molecular size to mature beta-toxin (33.2 kDa vs 34.8 kDa), but two share limited sequence identity (38%): phylogenetic analysis indicated that NetB is clearly a distinct toxin which does not belong to the beta-toxin clade (Keyburn et al., 2008). NetB was only identified in the majority (14 out of 18) of *C. perfringens* type A isolates from chickens suffering from NE, but not in 32 isolates which comprised those originating from birds not having the disease and from humans, pigs, cattle and sheep (Keyburn et al., 2008). However, in the initial research on NetB, 4 out of 18 strains capable of causing NE proved negative for the presence of netB in a PCR assay and did not produce NetB, which was confirmed in a Western blot test. Thus, it was suggested that in some strains NetB is not an essential component of virulence (Keyburn et al. 2008). By contrast, in Iran, the netB gene was first detected in a small percentage (7.77%) in chickens with NE in organic broiler farms (Ezatkhah et al., 2016**).**

Johansson et al. (2010) used PCR to investigate into the genetic diversity and prevalence of the gene coding for NetB toxin in *Clostridium perfringens* isolated from a broiler flock affected by mild NE in Sweden. Out of the 34 isolates from NE lesions in 18 birds, 31 (91%) were found to be positive for *NetB* gene (Johansson et al., 2010). However, out of the 23 isolates taken from the stomach and caecums of six birds without NE lesions, in 16 (70%) the *NetB*

gene was confirmed. It was concluded that 1) mild NE can be associated with NetB, but not with the specific *C. perfringens* genotype, 2) NetB can easily be transmitted between various genotypes, and 3) that some other virulence factors may decide whether a severe NE disease will develop or not, for instance, it can be the ability of certain strains to inhibit the growth of less pathogenic or apathogenic *C. perfringens* strains and establish dominance and lead to severe NE disease (Johansson et al., 2010). Continuing their research, Keyburn et al. (2010) assessed 44 isolates obtained from chickens with NE from Australia, Europe (Belgium and Denmark) and Canada and 55 ones from healthy Australian and Belgian chickens. As many as 70% of the affected birds were found positive for the NetB toxin, which highly correlated with the presence of netB gene. However, 2 out of the 55 healthy chickens' isolates were carriers of the gene. In addition, it was revealed that netB is highly conserved. However, the gene coding for TpeL toxin was detected in a few NetB-positive isolates from diseased chickens. NetB-negative isolates, obtained from affected birds, failed to produce NE in experimental conditions. Thus, it was proven that NetB is important in pathogenesis.

The first research into the occurrence of the toxin gene (netB) in *C. perfringens* isolates outside Australia was conducted by Martin and Smyth (2009). They inspected 106 American isolates of *C. perfringens* (92 from chickens and 8 from cattle). The netB gene was confirmed in 14 chicken isolates: 7 originated from those with NE, and 7 from non-affected ones, but also in one isolate obtained from a 3-year-old cow with liver abscesses. This is considered to be the first detection of NetB gene in isolates in a non-chicken *C. perfringens* isolate. However, five isolates, each taken from one chicken with NE, were negative for netB gene, as were another 24 isolates recovered from one of these diseased birds. Based on their results, the authors suggest that the importance of NetB for the onset of NE requires additional research and involves assessing the disease-producing capability of both netB-positive strains from healthy chickens, and those netB-negative isolated from diseased birds (Martin and Smyth, 2009). The detection of the netB gene in isolates from healthy birds (in 8.8%) implies that its presence is not sufficient to cause disease, and points to the importance of predisposing factors for the development of NE (Paiva and McElroy, 2014).

It was proven experimentally that TpeL toxin (an acronym which stands for toxin *C. perfringens* large cytotoxin encoded by the *tpeL* gene) may be a significant virulence factor in the development of NE (Coursodon et al., 2012). TpeL belongs to the large clostridial toxins group (LCTs), which is produced by minimum four pathogenic clostridium species: *C. difficile* (TcdA and TcdB

toxins), *C. sordellii* (TcsH and TcsL), *C. novyi* (TcnA) and TpeL *Clostridium perfringens* (TpeL) (Chen and McClane, 2015). TpeL was discovered in *C. perfringens* type C strains, but also in ATCC 3626, a type B strain (Amimoto et al., 2007). Its molecular mass was 191kDa (Amimoto et al., 2017). Some recent research discovered that many type C strains, and nearly all type B strains carry *TpeL* toxin gene, which is often (but not always) located near the *cpb* gene on plasmids of 90 kb or 60 to 65kb (Chen and McClane, 2015). TpeL possesses a glycosyltransferase activity, but is sensitive to trypsin, which is important to the pathogenesis of NE (Amimoto et al., 2007; Chen and McClane, 2015). The cytotoxicity of TpeL was proven on Vero, HeLa and rat pheochromocytoma PC12 cell cultures (Chen and McClane, 2015) and its lethality to mice (Amimoto et al., 2007). This finding implies a potential TpeL contribution to virulence, but this still remains to be proven in animal models (Chen and McClane, 2015). *TpeL* gene was detected in isolates of *C. perfringens* type A possessing also the NetB gene, which suggests that both TpeL-positive strains are associated with NE, although it is considered that the primary role in the pathogenesis is played by NetB toxin (Coursodon et al., 2012; Bailey et al., 2013). TpeL is probably produced also in the guts during clostridium diseases and contributes to type B and C infections in hosts with decreased trypsin levels due to disease, diet or age (Chen and McClane, 2015). It was proven experimentally that TpeL potentiates the effect of other virulence characteristics of NE strains of *C. perfringens* (Coursodon et al., 2012). The virulence of TpeL-positive and -negative *C. perfringens* strains isolated from chicks with NE was examined in healthy birds (Coursodon et al., 2012). Gross lesions typical of NE were observed in all artificially infected birds, but were more prominent in those inoculated with positive strains in comparison with their counterparts inoculated with a negative strain. It was also proven that infection with TpeL-positive strains may produce a disease with a more rapid course and with higher mortality (Coursodon et al., 2012). Although a multiplex PCR has been established for the detection of netB and tpeL genes (Bailey et al., 2013), relatively few bacterial populations have been screened for these two genes (Bailey et al., 2013).

Thus, the lack of extensive epidemiological studies render the global distribution of this newly discovered toxin and its connection with NE unknown (Bailey et al., 2013). It is possible that other virulence factors, such as hydrolytic enzymes and some unidentified toxins may contribute to the pathogenesis of NE (Van Imersseel et al. 2008). Combat against NE in intensive poultry production can be aided by immunization with CPA, NetB or some other proteins, administered conventionally or using recombinant attenuated *Sal-*

monella vectors. Inevitably, progress should be based mainly on genomic and proteomic analyses (Coper and Songer, 2009).

CONCLUDING CONSIDERATION

In the end, it should be kept in mind that modern poultry industry means intensive production whose object is a live system/organism. It is aimed at generating profit, in other words, to gaining maximum quantity of poultry meat in short time at as low cost as possible. However, the production takes place in conditions which are mainly in stark contrast with the lifestyle to which the poultry have been evolutionary adapted to: in closed, overpopulated housing, in the environment with high concentration of causative agents and permanent influence of a battery of factors predisposing to ailments, which are practically impossible to control. Research efforts that aim for casting light on the mechanisms of pathogenesis (which is still to a great extent unclear) are targeted at the choice of potent immunogenes and the improvement in immunoprophylaxis. The question remains open: in those conditions how is it possible to sustain health and the integrity of the intestinal mucosa, the microbiome of the digestive system, and the full potentials of the local gut immunity and the systemic immune response? Thus, we consider that the industrialization of live organisms, in this case of poultry production, will inevitably still be taking its toll: subclinical and acute forms of NE will still occur.

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