Letters in Applied Microbiology ISSN 0266-8254

ORIGINAL ARTICLE

In vitro inhibitory effect of hen egg white lysozyme on *Clostridium perfringens* type A associated with broiler necrotic enteritis and its α-toxin production

G. Zhang, S. Darius, S.R. Smith and S.J. Ritchie

Inovatech Bioproducts, Abbotsford, BC V2T 6K8, Canada

Keywords
α-toxin, *Clostridium perfringens*, ELISA, lysozyme, necrotic enteritis.

Correspondence
Guopeng Zhang, Inovatech Bioproducts, 31212 Peardonville Rd, Abbotsford, BC V2T 6K8, Canada. E-mail: jzhang@inovatech.com

2005/0210: received 1 March 2005, revised 2 September 2005 and accepted 5 September 2005

doi:10.1111/j.1472-765X.2005.01812.x

Abstract

Aims: *Clostridium perfringens* type A causes both clinical and subclinical forms of necrotic enteritis in domestic avian species. In this study the inhibitory effect of hen egg white lysozyme on the vegetative form of *Cl. perfringens* type A and the production of α-toxin *in vitro* was investigated.

Methods and Results: A micro-broth dilution assay was used to evaluate the minimal inhibitory concentrations (MIC) of lysozyme against three clinical isolates of *Cl. perfringens* type A in 96-well microtitre plates. The MIC of lysozyme against *Cl. perfringens* isolates was found to be 156 μg ml^{-1}. Scanning electron micrographs of the cells treated with 100 μg ml^{-1} of lysozyme revealed extensive cell wall damage. A quantitative sandwich ELISA for α-toxin produced by *Cl. perfringens* was developed based on a commercial ELISA kit allowing only qualitative detection. Addition of 50 μg ml^{-1} of lysozyme did not inhibit the growth of *Cl. perfringens* but significantly inhibited the toxin production.

Conclusions: Lysozyme inhibited the growth of *Cl. perfringens* type A at 156 μg ml^{-1}. At sublethal levels, lysozyme was able to inhibit the α-toxin production.

Significance and Impact of Study: Inhibition of *Cl. perfringens* type A and its α-toxin production by hen egg white lysozyme had never previously been reported. By inhibiting this avian pathogen and its toxin production, lysozyme showed potential for use in the treatment and prevention of necrotic enteritis and other *Cl. perfringens* type A related animal diseases.

Introduction

Clostridium (*Cl.*) *perfringens* is a Gram-positive, obligately anaerobic, spore-forming rod. According to the type of lethal toxins produced it has been classified into five different types (Cato *et al.* 1986). *Clostridium perfringens* type A, which produces primarily α-toxin (phospholipase C) is the major group associated with necrotic enteritis (NE; Wages and Opengart 2003b), cholangiohepatitis (Onderka *et al.* 1990) and gangrenous dermatitis (Wages and Opengart 2003a) in chickens.

Clostridial necrotic enteritis was first described by Parish (1961). Since then, this disease has been reported in most areas of the world where broilers are produced under intensive management conditions, and it has had significant economic impact on the poultry industry (Van der Sluis 2000). Acute clinical forms of the disease involve increased mortality and gross lesions consisting of large areas of necrosis of the lining of the lower small intestine and in some cases the caeca and liver (Wages and Opengart 2003b). A subclinical form has been associated with increased feed conversion ratio and retarded growth rate in birds (Kaldhusdal and Hofshagen 1992).

A variety of antibiotic growth enhancers such as virginiamycin and bacitracin have been used in feed to effectively control the incidence of NE. However, prophylactic applications of antibiotics are thought to induce antibiotic-resistant strains of human pathogens (Emborg *et al.* 2004). A number of European countries have banned the use of antibiotic growth promotants, including virginiamycin and

bacitracin. Similar actions may soon be seen in some other parts of the world because of the increasing public awareness of the possible negative impact of antibiotics on the environment and human health. It is believed that one serious problem faced by poultry producers in the absence of antibiotics will be an increase in NE (Newman 2000). In recent years, alternative approaches in addition to flock management strategies have been avidly pursued by poultry health personnel in order to reduce or eliminate the use of antibiotics in feed. New compounds from natural sources, depending on their efficacy against *Cl. perfringens* may be developed to replace antibiotics as growth promotants. Hen egg white lysozyme, a natural antimicrobial protein, could fill this need.

Lysozyme (EC 3.2.1.17, muramidase) occurs in a number of animal secretions and is considered an important component of the innate immune system. A recent study on the effect of *Eimeria tenella* and *Cl. perfringens* infection on the blood lysozyme levels in broiler chickens showed clear correlation between the lysozyme concentration and the severity of infection (Sotirov and Koinarski 2003). Because of its abundance in egg white, lysozyme is commercially extracted from hen eggs. Lysozyme is a 14·6 kDa single peptide that can result in cell lysis by cleaving the $\beta(1–4)$ glycosidic linkages between *N*-acetylmuramic acid and *N*-acetylglucosamine in the peptidoglycan layer of the bacterial cell wall (Proctor and Cunningham 1988). Lysozyme has an extremely high isoelectric point (>10) and consequently is highly cationic at neutral or acidic pH. In solution, lysozyme is stable at low pH and is active over a temperature range from 1°C to near boiling (Charter and Lagarde 1999).

Lysozyme has antimicrobial activity against many Gram-positive bacteria including some *Clostridium* species. It has been successfully used in the cheese industry as a bio-protectant for >20 years to control *Clostridium tyrobutyricum* that causes the 'late blowing' of hard and semi-hard cheeses as a result of butyric fermentation (Carini *et al.* 1985). To date, there are limited reports on the inhibitory effect of lysozyme against *Cl. perfringens* (Osa *et al.* 1990). To the authors' knowledge, the antimicrobial activity of lysozyme against isolates of *Cl. perfringens* type A associated with broiler NE has never been reported. The aim of this study was to investigate the inhibitory efficacy of hen egg white lysozyme against NE related *Cl. perfringens* and production of α-toxin.

Materials and methods

Lysozyme

Lysozyme was extracted and purified from hen egg white (Inovatech Bioproducts, Abbotsford, BC, Canada). It is a white powder with an enzymatic activity of 24 000 units mg^{-1} as defined by Shugar (1952). The lysozyme used in this study was confirmed for the absence of protease activity by azocoll assay (Calbiochem, La Jolla, CA, USA).

Clostridium perfringens isolates

Three *Cl. perfringens* type A strains associated with severe NE were tested for lysozyme MIC. Strains IM248 and IM400 were obtained from Animal Industry Branch, Ministry of Agriculture and Food, Province of British Columbia. Strain IM378 was kindly provided by Dr Charles L. Hofacre, University of Georgia, Athens, GA, USA. Only strain IM248 was studied for the α-toxin production.

Determination of MIC

A micro-broth dilution assay was used to evaluate the antimicrobial efficacy of lysozyme against the three *Cl. perfringens* strains. Sterile 96-well polystyrene microtitre plates with well capacities of 300 μl were used (Falcon, Becton Dickinson and Company, Franklin Lakes, NJ, USA) and 100 μl of fresh Luria broth (LB) was added to each well of the plate except for the first column. The pH of the medium was adjusted to 6·5 to simulate the intestinal environment (Sturkie 1999). Lysozyme stock solutions at 10 000 μg ml^{-1} were filter-sterilized by passage through a 0·45 μm membrane filter (Corning Incorporated, Corning, NY, USA). Two hundred microlitres of the lysozyme stock solution was added to each well of the first column using a multi-channel pipettor (Eppendorf, Hamburg, Germany). Then 100 μl of the stock solution was removed from the first column and mixed thoroughly with the broth in the corresponding wells of the second column six times. Subsequently, a 100-μl aliquot was removed from each well in this column and mixed with the corresponding well of the next column. This doubling dilution was performed in rows across the plate except for the last column that was kept for use as controls. This dilution procedure resulted with a gradient of lysozyme concentrations from 0 to 10 mg ml^{-1} across the plate. Ten microlitres of *Cl. perfringens* culture after overnight incubation was inoculated in each well of the plate to yield a final concentration of 10^5 CFU ml^{-1}. The microtitre plate was incubated in an anaerobic jar (BBL, Becton Dickinson and Company, Sparks, MD, USA) at 41°C for 24 h. Bacterial growth was measured by a change in absorbance at 560 nm using an automated microplate reader (Labsystems, Multiskan MS 3.0, Vantaa, Finland). The MIC was determined as the lowest lysozyme concentration that resulted in inhibition of *Cl. perfringens* growth (lack of increase in absorbance reading). Five microlitres of the

contents in those wells was also spotted on Shahidi–Ferguson Perfringens Agar (SFP, Oxoid, Basingstoke, UK) for confirmation of inhibition (absence of growth). The MIC assay was carried out in duplicate for each isolate and the assay was also repeated three times on different occasions. The inhibitory effect of lysozyme on IM248 was also evaluated under a scanning electron microscope (SEM). For the sample preparation, tubes of 10 ml of LB broth supplemented with 0 and 100 μg ml^{-1} of lysozyme were inoculated with 10^6 CFU ml^{-1} of fresh *Cl. perfringens* culture. The tubes were incubated anaerobically at 41°C over night. After incubation, the cells were spun down at 5000 ***g*** for 20 min, washed in PBS and resuspended in PBS supplemented with 2·5% fresh glutaraldehyde (Sigma, St Louis, MO, USA) at 41°C for 30 min. The tubes were then transferred to the BioImaging Facility, University of British Columbia, Vancouver at ambient temperature. The method for preparing SEM pictures was described at http://www.emlab.ubc.ca/p_mwave2.htm#protocols 2004.11.11. (Dr E. Humphrey, personal communication).

Development of a quantitative ELISA for α-toxin detection

The Bio-X α-toxin ELISA kit (Bio-X Diagnostics SPRLu, Jemelle, Belgium) was used to develop a quantitative assay for the α-toxin produced by *Cl. perfringens*. Appropriate dilutions of α-toxin standard (phospholipase C type XIV, Product No. P4039, Sigma, St Louis, MO, USA) or culture supernatant were added at 100 μl per well to both the wells coated with α-toxin antibody and the wells coated with nonspecific antibodies (blank). The dilutions were made with buffer provided in the kit. The sandwich ELISA procedure was followed according to the user's manual. The net absorbance readings were obtained by subtracting the absorbance of the blank from the absorbance of the samples. A standard curve was constructed based on doubling serial dilutions of the α-toxin standard (0–18·9 μg ml^{-1}) and their corresponding net absorbance values.

Inhibition of α-toxin production by lysozyme

An overnight culture of *Cl. perfringens* IM248 was inoculated at 10^5 CFU ml^{-1} into test tubes containing 10 ml LB supplemented with 0, 50 and 200 μg ml^{-1} lysozyme. After anaerobic incubation at 41°C for 18 h, both the bacterial numbers and α-toxin concentrations were quantified. The enumeration of *Cl. perfringens* was performed by serial 10-fold dilution in 0·1% peptone and pour-plating on SFP agar. The plates were incubated anaerobically for 18 h at 37°C before enumeration. For α-toxin analysis, the supernatant of cell suspensions (after centrifugation at 10 000 ***g*** for 15 min) from each tube was passed through a 0·45-μm membrane filter before the ELISA. All of the tests were carried out in triplicate.

Statistical analysis

Statistical analysis was carried out using SigmaStat (SPSS science, Version 2.0, 1997, Chicago, IL, USA). One-way ANOVA was used to analyse the effect of lysozyme on bacterial growth and α-toxin concentration. When a significant effect was detected, Tukey's test was used for pairwise comparison of the means to detect differences among treatments ($P < 0·05$).

Results

Lysozyme MIC

The MIC of lysozyme was determined as 156 μg ml^{-1} for all of the three *Cl. perfringens* type A strains tested in LB. The replicate tests on individual strains also gave the same MIC value. No growth (black colonies on SFP agar) was observed in wells supplemented with 156 μg ml^{-1} lysozyme. However, growth was evident in the wells with 0–78 μg ml^{-1} lysozyme. The detrimental effect of lysozyme on the cells of *Cl. perfringens* IM248 was also observed under a scanning electron microscope (Fig. 1). Cells in the untreated sample appeared to be healthy and intact. In contrast, extensive cell wall damage (large holes) was observed in samples treated with 100 μg ml^{-1} lysozyme.

Quantitative detection of α-toxin

The plot of net A_{450} and α-toxin concentration showed a typical polynomial/logarithmic curve with a detection end point below 18 ng ml^{-1} (Fig. 2). It was found that the data points with net $A_{450} < 1·2$ fitted a quadratic model well with a coefficient of relevance equal to 0·9997 (Fig. 2). According to the equation generated with Microsoft Excel:

$$y = -2·4289x^2 + 3·3877x + 0·0225 \quad (1)$$

where y and x are the net A_{450} value and α-toxin concentration, respectively, the α-toxin concentration (μg ml^{-1}) can be described in equation 2:

$$\alpha\text{-toxin concentration} = 0·70 - \sqrt{\frac{1·20 - net\text{A}}{2·4289}} \quad (2)$$

Therefore, a quantitative ELISA method is now available for α-toxin detection based on a given preparation of toxin standard. For samples with net A_{450} readings >1·20, further dilution is necessary.

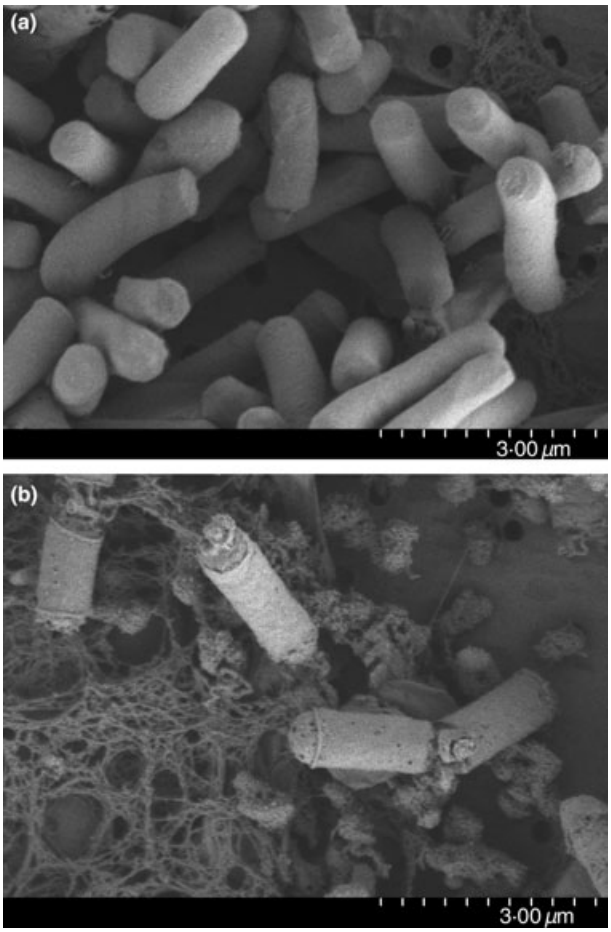

Figure 1 Scanning electron micrographs of *Clostridium perfringens* IM248 control and IM248 treated with 100 μg ml^{-1} of lysozyme in LB broth [L1, control (a), L2, lysozyme (b)].

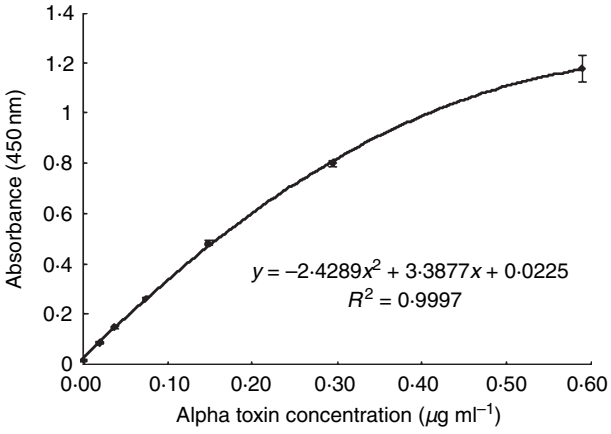

Figure 2 Quadratic regression curve showing the fit between OD values and α-toxin concentrations from 0 to 0·60 μg ml^{-1}.

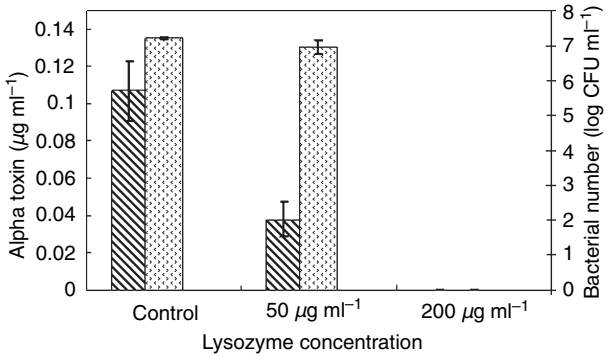

Figure 3 Inhibition of α-toxin production by *Clostridium perfringens* IM248 in the presence of sublethal level of lysozyme. ▨, α-toxin concentration; ▩, bacterial number.

Inhibition of α-toxin production by lysozyme

The ability of lysozyme to inhibit *in situ* α-toxin production by *Cl. perfringens* is shown in Fig. 3. Approximately 0·1 μg ml^{-1} α-toxin was produced in the control culture. In the presence of 50 μg ml^{-1} of lysozyme, although bacterial growth was not significantly affected the α-toxin level was significantly lower than in the control where no lysozyme was added ($P < 0·05$). An amount of 200 μg ml^{-1} of lysozyme completely inhibited the growth of *Cl. perfringens* and there was also no α-toxin detected in the culture supernatant.

Discussion

Although lysozyme's ability to inhibit several other *Clostridium* species including *Cl. tyrobutyricum*, *Cl. thermosaccharolyticum* and *Cl. botulinum* is documented (Carini *et al.* 1985; Hughey and Johnson 1987), there are limited reports on the inhibitory effect of lysozyme against *Cl. perfringens*. It is well-known that lysozyme does not kill bacterial endospores because of their unique capsule structure but it works to stimulate spores to germinate into vegetative cells (Lewis 1969). Lysozyme at <10 μg ml^{-1} has been successfully used in culture medium to facilitate the recovery of injured *Cl. perfringens* spores (Labbe and Chang 1995). According to the Bergey's Manual (Cato *et al.* 1986) as well as our own observation, *Cl. perfringens*, unlike most other clostridial species does not sporulate readily in ordinary culture media. To produce profuse sporulation, specially formulated media containing quinoline are necessary (Phillips 1986). Lysozyme was found able to inhibit *Cl. perfringens* growth at 156 μg ml^{-1} in LB and the electron photomicrographs also clearly demonstrated the destructive effect of lysozyme on *Cl. perfringens* vegetative cells.

The conventional assays of α-toxin are based on the measurement of phospholipase activity. However, these

assays are generally difficult to perform and the sensitivity is inferior to ELISA (Kurioka and Matsuda 1976; Hale and Stiles 1999). Also, samples such as gut content and culture supernatant might be associated or contaminated with other hydrolytic enzymes, which could be confused with or inhibit *Cl. perfringens* phospholipase C activity. A number of quantitative ELISA assays for α-toxin have been reported in recent years (Liu and Blaschek 1996; Hale and Stiles 1999). However, these methods were developed at the individual laboratories with noncommercial antibodies or conjugates and thus make the assay difficult to repeat. The commercially available α-toxin ELISA kit (Bio-X Diagnostics) can only allow qualitative detection. This paper describes a method to quantify the α-toxin based on the easily accessible Bio-X ELISA kit. The value of this ELISA kit as a quantitative procedure was demonstrated with a partially purified α-toxin standard from Sigma. Therefore the calculated α-toxin levels in the samples are relative values to this standard. In this study approx. 0·1 $\mu g\ ml^{-1}$ of α-toxin was detected in overnight cultures of *Cl. perfringens* IM248, which is in agreement with the concentration range of different strains tested by other workers (Hale and Stiles 1999).

The results in this *in vitro* study suggest that lysozyme not only can kill the bacterial pathogen but can inhibit production of α-toxin, the causative agent for gross lesions in the disease of NE, even at sublethal levels. However, cage and floor pen trials using an appropriate challenge model need to be conducted to further evaluate the efficacy of lysozyme *in vivo* against NE caused by *Cl. perfringens* type A in comparison with antibiotic growth promotants. As hen egg white lysozyme is a very stable product it can be blended with feed in powder form or dissolved in drinking water. If the results from *in vivo* studies are positive, synergies between lysozyme and other natural products may also be sought to produce cost-competitive blends for NE prevention.

Acknowledgement

The funds provided by Investment Agriculture Foundation of British Columbia are acknowledged.

References

Carini, S., Mucchetti, G. and Neviani, E. (1985) Antimicrobial activity of lysozyme against clostridia and use in cheese production – a review. *Microbiol Alimen Nutr* **3**, 299–320.

Cato, E.P., George, W.L. and Finegold, S.M. (1986) Genus *Clostridium* Prazmowski 1880. In *Bergey's Manual of Systematic Bacteriology*, vol. 2 ed. Sneath, P.H.A., Mair, N.S., Sharpe, M.E. and Holt, J.G. pp. 1141–1200. Baltimore, MD: Lippincott Williams and Wilkins.

Charter, E.A. and Lagarde, G. (1999) Lysozyme and other proteins in eggs. In *Encyclopedia of Food Microbiology* ed. Batt, C.A., Robinson, R.K., Patel, P. and Patel, P.D. pp. 1582–1587. Adelaide, London and New York: Academic Press.

Emborg, H.D., Andersen, J.S., Seyfarth, A.M. and Wegener, H.C. (2004) Relations between the consumption of antimicrobial growth promoters and the occurrence of resistance among *Enterococcus faecium* isolated from broilers. *Epidemiol Infect* **132**, 95–105.

Hale, M.L. and Stiles, B.G. (1999) Detection of *Clostridium perfringens* alpha toxin using a capture antibody ELISA. *Toxicon* **37**, 471–484.

Hughey, V.L. and Johnson, E.A. (1987) Antimicrobial activity of lysozyme against bacteria involved in food spoilage and food-borne disease. *Appl Environ Microbiol* **53**, 2165–2170.

Kaldhusdal, M. and Hofshagen, M. (1992) Barley inclusion and avoparcin supplementation in broiler diets. 2. Clinical, pathological, and bacteriological findings in a mild form of necrotic enteritis. *Poult Sci* **71**, 1145–1153.

Kurioka, S. and Matsuda, M. (1976) Phospholipase C assay using *p*-nitrophenylphosphoryl choline together with sorbitol and its application to studying the metal and detergent requirement of the enzyme. *Anal Biochem* **75**, 281–289.

Labbe, R.G. and Chang, C.-A. (1995) Recovery of heat-injured spores of *Clostridium perfringens* type B, C and D by lysozyme and an initiation protein. *Lett Appl Microbiol* **21**, 302–306.

Lewis, J.C. (1969) Dormancy. In *The Bacterial Spore* ed. Gould, G.W. and Hurst, A. pp. 301–358. London and New York: Academic Press.

Liu, Z. and Blaschek, H.P. (1996) Monoclonal antibody-based ELISA for detection of *Clostridium perfringens* alpha-toxin. *J Food Protect* **59**, 621–625.

Newman, L.J. (2000) *Necrotic Enteritis: Managing Without Antibiotics*. Factsheet No. 120. Poultry Industry Council's Poultry Health Conference, Guelph, ON.

Onderka, D.K., Langevin, C.C. and Hanson, J.A. (1990) Fibrosing cholehepatitis in broiler chickens induced by bile duct ligations or inoculation of *Clostridium perfringens*. *Can J Vet Res* **54**, 285–290.

Osa, J.M., Benezet, A., Botas, M., Olmo, N. and Florez, F.P. (1990) Action of commercial lysozyme (chlorhydrate) on certain bacterial species. *Alimentaria* **27**, 49–59.

Parish, W.E. (1961) Necrotic enteritis in the fowl (*Gallus gallus domesticus*). I. Histopathology of the disease and isolation of a strain of *Clostridium welchii*. *J Comp Pathol* **71**, 391–404.

Phillips, K.D. (1986) A sporulation medium for *Clostridium perfringens*. *Lett Appl Microbiol* **3**, 77–79.

Proctor, V.A. and Cunningham, F.F. (1988) The chemistry of lysozyme and its use as a food preservative and a pharmaceutical. *Crit Rev Food Sci Nutr* **26**, 359–395.

Shugar, D. (1952) The measurement of lysozyme activity and the ultra-violet inactivation of lysozyme. *Biochim Biophys Acta* **8**, 302–309.

Sotirov, L. and Koinarski, V. (2003) Lysozyme and complement activities in broiler chickens with coccidiosis. *Revue Méd Vét* **154**, 780–784.

Sturkie, P.D. (1999) Secretion of gastric and pancreatic juice, pH of tract, digestion in alimentary canal, liver and bile, and absorption. In *Avian Physiology*, 5th edn ed. Whittow, G.C. pp. 197–209. New York: Academic Press.

Van der Sluis, W. (2000) Necrotic enteritis (3): clostridial enteritis is an often underestimated problem. *World poult* **16**, 42–43.

Wages, D.P. and Opengart, K. (2003a) Gangrenous dermatitis. In *Diseases of Poultry*, 11th edn ed. Saif, Y.M. pp. 791–795. Ames, IA: Iowa State Press.

Wages, D.P. and Opengart, K. (2003b) Necrotic enteritis. In *Diseases of Poultry*, 11th edn ed. Saif, Y.M. pp. 781–785. Ames, IA: Iowa State Press.