BRIEF COMMUNICATION: Chitosan is a highly effective *in vitro* antibacterial agent against the strains of bacteria causing footrot, but is not effective in treating stage-four footrot on farm.

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Introduction

Footrot affects sheep, deer, cattle and goats. It is caused by a mixed bacterial infection of *Fusobacterium necrophorum* and *Dichelobacter nodosus*. Footrot is highly contagious and spreads rapidly in warm and moist conditions. Virulent footrot results in the destruction of hoof tissue resulting in reduced animal weight, fertility and wool production. If untreated, footrot can lead to a slow and painful death (Kennan et al. 2011). Nationally, footrot is estimated to cost $80–100M pa in lost production (Greer, 2005; Hickford et al. 2005).

Chitin is an abundant waste product from the seafood industry that can be converted to biodegradable chitosan, a compound that has proven biomedical antimicrobial and haemostatic/wound healing properties (Vinsova &Vavrikova 2008). NZ has a unique abundance of chitin as a waste material from its fishing industry (squid pens and crab shells). By coupling this cheap source of raw material with minimal processing to derive chitosan, a cost-effective method to prevent and/or treat footrot compared to using vaccines, zinc sulphate footbaths and antibiotic treatment and without the trait trade-offs inherent with genetic-selection approaches is possible. We have previously reported research, using chitosan derived from snowcrab, that demonstrated high *in vitro* antibacterial activity of chitosan derivatives against *D. nodosus* and *F. necrophorum* (Mros et al. 2012). The present study was aimed at investigating the efficacy of chitosan at higher concentrations for the treatment of footrot in merino sheep under normal farming conditions.

Materials and methods

Chitosan

Alaska snow crab was obtained from Waseta International (Shanghai, China). A chitosan derivative was prepared that had 75% deacetylation degree and was dissolved in 1% acetic acid to prepare a 10% (w/w) solution that was diluted with water for use on farm.

Bacterial strains and minimum inhibitory concentration assays

Strains of bacteria associated with the pathogenesis of footrot *Dichelobacter nodosus* (Wallaceville, AgResearch Centre) and *Fusobacterium necrophorum* (NCTC 10575, DSM 20698) Type A were grown as described earlier (Mros et al. 2012).

The microplate method described by Eloff (1998) was used to determine the inhibitory concentrations of chitosan samples against *D. nodosus and F. necrophorum*. A 10µl aliquot of bacterial culture (approximately 10^8 cfu/ml) suspended in growth medium was added to wells in a 96-well microplate. Chitosan dissolved in 1% acetic acid (100-µl) was placed in the first well and serial dilutions were made resulting in a final concentration range from 0.5 mg/ml down to 0.0078 mg/ml (Mros et al, 2102). Chitosan-free 1% acetic acid was used as a blank control. The therapeutic antibiotic streptomycin sulphate (Sigma) was used as a positive control at concentrations ranging from 1.02 mg/ml to 0.015 mg/ml. Microplates were incubated overnight at 37°C in an AnaeroPack System™. A 20µl aliquot of p-iodonitro-tetrazolium violet (INT), dissolved in water at a concentration of 0.4%w/v, was added to the wells and plates were incubated at 37°C for 30 minutes. The colourless salt is reduced to a red-coloured formazan product by biologically active organisms. Inhibition values were recorded as the lowest concentration of chitosan that inhibited bacterial growth. A 10-µl aliquot of each dilution, following the INT assay, was cultured onto Wilkens-Chalgren agar to determine if the inhibitory concentration was bactericidal.

Animal trial

The animal trial was carried out on a farm in Middlemarch (Otago, South Island) from the 14th October 2013 until the 8th of November 2013 with permission of University of Otago Animal Ethics committee (AEC 17/13). A veterinarian selected forty merino sheep having at least one foot with grade-four footrot from a flock of 400 animals with footrot (Mulvaney 2002). The animals were ear tagged and randomly assigned into four treatment groups (untreated control, $ZnSO_4$ bath treatment, 1 mg/ml chitosan and 10 mg/ml chitosan bath treatments). For ease of identification, each group had a different coloured dye sprayed on its head and the sheep were then weighed. All the sheep were run together in a paddock with good feed. Sheep were foot bathed on the 14th, 21st, 28th of October and 4th of November. On the final day of the trial, the sheep were assessed by the veterinarian for the grade of footrot in each foot and were treated with antibiotics if required and then weighed.

Statistical analysis

A student test (t-test) was used to test differences between the MIC values for chitosan and streptomycin sulphate. Analysis of variance (ANOVA) was carried out using Minitab (version 16.2.4). A general linear model was used to determine the effects of treatments on weight gain and footrot scores. Significant differences among mean values were determined at a 5% significance level.

Figure 1. Mean weight gain (mean ± SEM) of sheep with grade-four footrot randomly assigned to four experimental groups of ten animals each. The animals were foot bathed in either zinc sulphate, 1 mg/ml chitosan or 10 mg/ml chitosan solutions four times over the 24-day trial or left untreated. The sheep were weighed before and after 24 days of treatment.

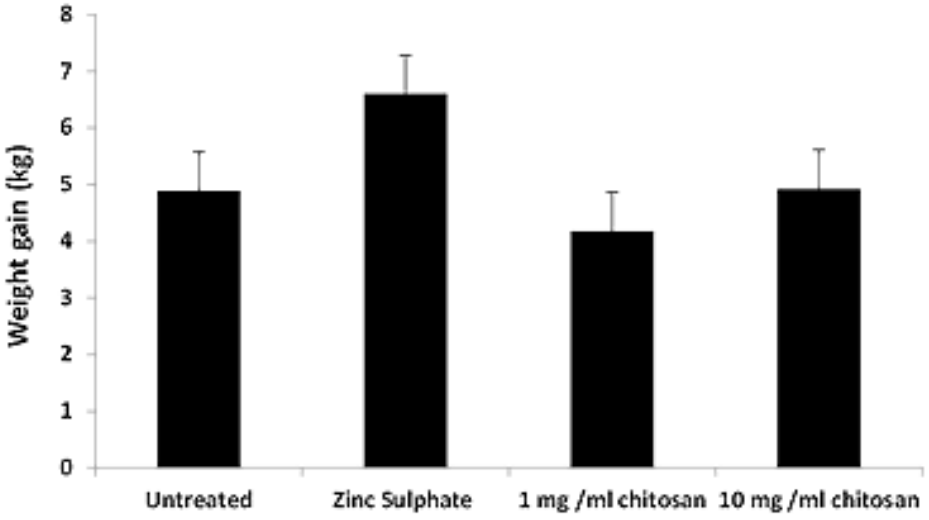
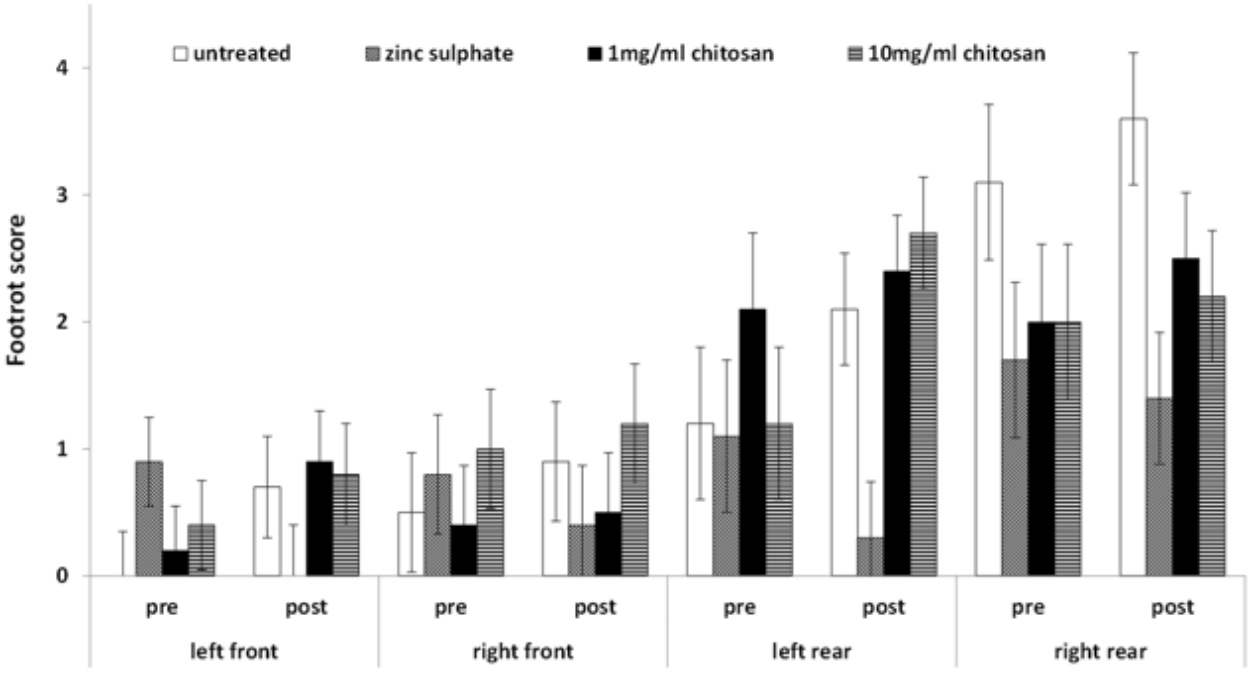


Figure 2. Footrot scores of sheep feet used in the present study. Results are expressed as mean score (± SEM) for footrot infection in sheep with grade-four footrot randomly assigned to four experimental groups of ten animals each before and after 4 days of treatment. The animals were foot bathed in either zinc sulphate. 1 mg/ml chitosan or 10 mg/ml chitosan solutions four times over the 24-day treatment period or left untreated. Pre = pre -treatment and post = post-treatment.



Results

Antimicrobial activity of chitosan derivatives

The *in vitro* antimicrobial activity of the chitosan against the two bacterial strains involved in the pathogenesis of footrot, *D. nodosus* and *F. necrophorum showed no growth on agar at a concentration of* 0.0078 mg/ml and the positive control at 0.015mg/ml. Acetic acid (1%) did not inhibit growth of the bacteria.

Sheep treated with zinc sulphate had the highest weight gain (6.6 ± 0.69 kg) over the 24 days treatment period. This was significantly higher than for the sheep treated with 1 mg/ml of chitosan ($P < 0.05$). The average weights of sheep from untreated control and treated with10 mg/ml chitosan were not significantly different from either the zinc sulphate treated or the 1 mg/ml chitosan treated sheep (Figure 1). Only zinc sulphate treatment demonstrated any improvement in the treatment of footrot compared to the untreated group (Figure 2).

Discussion

Chitosan is a known antimicrobial agent effective against a wide range of organisms (Vinsova &Vavrikova 2008). We have previously reported the antimicrobial action of chitosan against the anaerobic organisms implicated in the pathogenesis of footrot (Mros et al. 2012) and the present study confirmed that activity using a freshly made chitosan preparation. The MICs of chitosan against the microorganisms that cause footrot were lower than for the therapeutic antibiotic streptomycin sulphate. Chitosan damages the cell membrane of *D. nodusus* causing a peripheral cytoplasm leakage that subsequently leads to the death of the microorganism (Mros et al. 2012). Under *in vitro* conditions, the damage can be observed within two hours of incubation of the chitosan with the organism. It is normal practice to increase the amount of a potentially therapeutic compound when taking it from an *in vitro* to an *in vivo* trial. In this instance amounts of 1 mg/ml and 10 mg/ml were chosen. However, these amounts were not high enough to have an effect on treating footrot in this trial even though the *in vitro* results were outstanding.

Environmental conditions (constant exposure to a wet contaminated environment) may have had an impact on this trial as well as running the untreated controls with the treated animals so that there was constant exposure to the microorganisms that cause footrot. However, this never-the-less mimics the necessary real-world performance conditions required for commercial application. Further on farm investigations by using either higher concentrations of chitosan, more frequent foot bathing or delivering the chitosan in a different formulation are required to determine how the high lab-based efficacy against footrot bacteria can be translated into high field-based efficacy.

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