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PRODUCTION AND MANAGEMENT: *Original Research*

Effects of increasing dietary zinc sulfate fed to gestating ewes: II. Milk somatic cell count, microbial populations, and fatty acid composition*

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ABSTRACT

Objective: The objective of the research was to evaluate the effects of increasing dietary Zn sulfate concentration for primiparous gestating ewes on subsequent milk SCC, intramammary microbial identifications, and fatty acid composition.

Materials and Methods: Commercial white-face (WF; n = 27) and black-face (BF; n = 24) ewes (age ≈18 mo; BW = 87.48 ± 8.37 kg) were sorted into breed-type groups and within groups ranked by BW, and then, they were randomly divided into 3 dietary supplement treatment groups: CON (n = 13; 40 mg/kg Zn; ≈1 × NASEM recommendations), Zn500 (n = 21; 500 mg/kg Zn; ≈4 × NASEM recommendations), and Zn1000 (n = 17; 1,000 mg/kg Zn; ≈7 × NASEM recommendations). Treatments were administered in Zn-fortified pelleted alfalfa (0.45 kg/ewe per day) and fed from 87.5 ± 8.9 d of gestation until parturition. Milk traits collected at parturition (d 1 of lactation), ≈30 d of lactation, and lamb weaning (≈90 d of lactation) were assessed as repeated measures with fixed effects of treatment, breed type, and litter size.

Results and Discussion: The treatment × breed type interaction affected ewe logSCC ($P = 0.01$), and within Zn500, BF had greater logSCC than WF ewes (5.90 ± 0.08 vs. 5.46 ± 0.08; $P < 0.01$). However, breed types did not differ between CON and Zn1000 treatments ($P \geq 0.92$). Ewe logSCC was greatest ($P < 0.01$) at weaning (6.03 ± 0.06), intermediate at parturition (5.72 ± 0.06; d 1), and least at d 30 of lactation (5.21 ± 0.06). Intramammary infections were common in milk samples collected at parturition (77%) and weaning (47%) based on culture-based microbial identifications. The most frequently identified species included *Bacillus* spp. and *Staphylococcus* spp. Black-face ewes had greater concentrations of C16:1 (1.78 mg/100 vs. 1.39 mg/100 mg of fatty acid per 100 mg of total fatty acids), C17:1 (0.86 mg/100 vs. 0.76 mg/100 mg of fatty acid per 100 mg of total fatty acids), and C20:4 (0.28 mg/100 vs. 0.24 mg/100 mg of fatty acid per 100 mg of total fatty acids; $P \leq 0.04$) than WF ewes.

Implications and Applications: Including Zn in diets beyond NASEM recommendations from mid to late gestation had no effect on ewe milk SCC, microbial pathogens identified, or fatty acid composition. However, findings indicated there may be important breed differences in dietary Zn utilization and requirements affecting intramammary inflammation.

Key words: gestation, mineral nutrition, sheep, subclinical mastitis, zinc

INTRODUCTION

In extensively and semi-extensively managed sheep populations in the western United States, forage Zn concentrations are lowest during the periods that coincide

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with breeding, gestation, and early lactation (Julian et al., 2020). Furthermore, it is estimated that 30 to 50% of extensively managed sheep operations in the western United States fail to consistently provide supplemental mineral (Page et al., 2018; Stewart et al., 2020). The companion research to the present found that feeding Zn to gestating primiparous ewes above NASEM (2007) recommendations did not improve indices of lamb performance but increased wool production and exerted differential effects in serum mineral element concentrations and progeny's neonatal concentrations between fine-wool and meat-type ewes (Stewart et al., 2020). Zinc's essential role in proper immune system function, and specifically in indices of mammary health, has been observed in dairy cattle when included at recommended dietary levels (Whitaker et al., 1997; Cope et al., 2009). Additionally, Zn has been shown to influence de novo fatty acid synthesis in dairy cattle (Wiking et al., 2008) and alter lipid membrane integrity by increasing unsaturated and longer chain fatty acid concentrations (Hermansen et al., 1995). Still, the effect of Zn supplementation above recommended NASEM (2007) levels on ewe mammary health and milk composition in extensive and semi-extensive sheep production is not well understood. Therefore, the objective of the current research was to determine the effects of increasing Zn sulfate ($ZnSO_4$) concentrations from mid to late gestation on subsequent milk SCC, mastitis microbial pathogens, and fatty acid composition.

MATERIALS AND METHODS

The experiment was reviewed and approved by the Institutional Animal Care and Use Committee at the University of Wyoming (#A-3216-01).

Study Design and Treatment Diet Administration

The experimental design was described in detail by Stewart et al. (2020). Briefly, a total of 78 ewes (age ≈18 mo; BW 87.48 ± 8.37 kg) of Rambouillet and Hampshire × Suffolk background [white face (**WF**) and black face (**BF**), respectively] were used in the experiment. Ewes were sorted into breed type groups and, within groups, ranked by BW and randomly divided into 3 dietary supplement treatment groups along the rank: **CON** (n = 27, 40 mg/kg Zn, ≈1 × NASEM recommendations), **Zn500** (n = 29, 500 mg/kg Zn, ≈4 × NASEM recommendations), and **Zn1000** (n = 24, 1,000 mg/kg Zn, ≈7 × NASEM recommendations). Each ewe received 0.45 kg/d of their respective Zn-fortified alfalfa pellet, which was dispensed from 2 mobile feed apparatuses (Super SmartFeeder; C-Lock Inc.) each with 4 feed pans. Therefore, ewes were managed in one contemporary group but could only gain access to their treatment group's feed pans, and ewe was the experimental unit. Ewes were acclimated to the Super SmartFeeder for 10 d in a 100 × 20 m enclosure with the CON alfalfa pellet. Total consumption of each treatment throughout the trial was used to determine mean daily intake of Zn and was as follows: CON = 104 mg/d; Zn500 = 462 mg/d; Zn1000 = 772 mg/d. Thus, according to NASEM (2007) factorial estimated daily Zn recommendations, diets met 100, 400, and 700% for CON, Zn500, and Zn1000 treatment groups, respectively.

Animal Management

Ewes were exposed to rams of their breed type in multiple-sire mating groups for 51 d, started the trial at 87.5 ± 8.85 d of gestation, and remained on their treatment until parturition. This corresponded to an average trial period of 61.3 ± 8.62 d. At the beginning of the trial (d 0–42), ewes were managed on 19.2-ha hay meadow pasture regrowth with meadow brome (*Bromus riparius*), timothy (*Phleum pretense*), and creeping foxtail (*Alopecurus arundinaceus*) as predominant grass species. Site selection emphasized a forage source deficient in Zn (17 mg/kg Zn; DM basis; Table 1). Ewes were allowed to graze until d 42 of the trial (132.7 ± 8.62 d gestation) and then moved to a 40 × 40 m housed facility where they were offered *ad libitum* access to meadow hay (21 mg/kg Zn; Table 1). All ewes were subject to the same management after parturition.

Data Collection

Milk Collection and SCC. Colostrum was collected between 12 and 18 h after parturition (d 1), and milk was collected at ≈30 d (mid-lactation) and ≈90 d (lamb weaning) after parturition. Before each sampling, teats were scrubbed using a 70% ethanol wipe, and ewes were manually milked with a gloved hand. One sample of approximately 20 mL of milk was collected from each udder half and combined into a single 50-mL conical tube and used for SCC analysis. An additional subsample was collected and frozen at −20°C until further analyzation for microbial identification or fatty acid composition. Samples for SCC testing were preserved with 8 mg of Bronopol and 0.3 mg of Natamycin (Microtabs II; D & F Control Systems Inc.) and put on ice and shipped to a participating laboratory (Montana State University) for analysis within 3 d of collection. Somatic cell count was quantified on a LactiCyte HD (Page & Pedersen International Ltd.) according to the manufacturer's protocol.

Microbial Analysis. Ewe colostrum and milk samples were analyzed for microbial identification. The thawed sample (1 mL) was pipetted into 1.5-mL microcentrifuge tubes and centrifuged at 5,000 × *g* for 5 min at room temperature to separate fat, supernatant liquid, and milk pellet. Fat and the majority of the supernatant were discarded, and the pellet was resuspended in the remaining supernatant (≈0.1 mL) via vortexing. Resuspended pellets were streaked onto compartmentalized plates (X-plate, Thermo Fischer Scientific Inc.) containing 1 each of 4 microbiological growth media: MacConkey agar (HiMedia Laboratories Pvt. Ltd.); Trypticase soy agar (**TSA**;

Becton, Dickinson and Company); TSA + 5% sheep blood agar (Hardy Diagnostics); and Sabouraud dextrose agar (Neogen Corporation) using a 10-μL inoculating loop. These media were chosen to select for the following targets: MacConkey agar suppresses the growth of Gram-positive bacteria, thereby favoring growth of Gram-negative and enteric bacilli; TSA is a general-purpose, nonselective medium for recovery of most bacteria present; TSA + 5% sheep blood media contains added nutrients and growth factors to improve recovery of fastidious bacteria in comparison to the base medium TSA; and Sabouraud dextrose agar is used to support the growth of fungi. Plates were incubated aerobically at 37°C for 18 to 24 h. The number of colonies and colony morphologies were recorded following the standard incubation period; however, plates that did not display microbial growth were reincubated for an additional 24 h to ensure that growth of slow growing and fastidious bacteria was captured by the procedure (Dohoo et al., 2011). Culture-positive plates were considered indicative of an intramammary infection.

Confirmation of all isolates was carried out via matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (**MALDI-TOF MS**). The direct colony transfer method was applied, which involves transfer of purified colonies in triplicate with a sterile 1-μL inoculating loop onto a 48-well steel-target plate for microbial identification. Subsequently, 1 μL of Matrix A CHCA (α-cyano-4-hycroxycinnamic acid) was added to the center of each well before identification was completed by using an Agena MassARRAY instrument (Agena Bioscience) and a spectrum match factor library. If none of the replicates yielded a successful match, samples were reanalyzed in triplicate.

Multiplex-PCR was used in parallel as a culture-independent method for microbial identification. Frozen milk samples containing sufficient volume for DNA extraction following culturing methods were subjected to microbial identification using a Thermo Scientific PathoProof Complete-16 kit with the Primer Mix 4 for Applied Biosystems 7500 and 7500 Fast instruments. This test kit identifies 15 possible taxa commonly isolated from cases of bovine mastitis, including *Corynebacterium bovis*, *Enterococcus* spp., *Escherichia coli*, *Klebsiella oxytoca* and *Klebsiella pneumoniae*, *Mycoplasma bovis*, *Mycoplasma* spp., *Prototheca* spp., *Serratia marcescens*, *Staphylococcus aureus*, *Staphylococcus* spp., *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, *Streptococcus uberis*, *Trueperella pyogenes* and *Peptoniphilus indolicus*, and many common yeasts. The kit also screened for the β-lactamase penicillin resistance gene in staphylococci, including in *Staph. aureus* and all major coagulase-negative staphylococci. Four PCR reactions were run for these milk samples (Eurofins DQCI LLC.).

Table 1. Chemical composition of zinc-fortified pelleted diets, meadow hay regrowth, and meadow hay

	Treatment[1]			Basal dietary component[2]	
Item	CON	Zn500	Zn1000	Meadow hay regrowth	Meadow hay
Nutrient composition					
DM, %	92.9	93.2	93.4	37.5	86.6
CP, %	20.4	20.9	20.2	6.8	11.2
NDF, %	38.5	37.2	38.6	70.7	54.2
ADF, %	31.9	30.5	34.0	46.5	33.4
Mineral composition					
Ca, %	1.24	1.29	1.21	0.82	0.59
P, %	0.28	0.31	0.28	0.13	0.23
K, %	3.03	3.13	3.14	0.82	0.59
S, %	0.32	0.37	0.38	0.14	0.22
Mg, %	0.33	0.34	0.31	0.18	0.21
Na, %	0.11	0.12	0.12	0.07	0.05
Fe, mg/kg	375.0	396.0	330.0	469.0	173.0
Mn, mg/kg	79.0	109.0	81.0	91.0	53.0
Cu, mg/kg	14.6	9.0	12.4	2.9	8.0
Zn, mg/kg	32.4	551.5	998.3	19.3	24.5
Mo, mg/kg	3.26	3.54	3.30	1.59	1.23

[1]CON = 104 mg of Zn/d, Zn500 = 462 mg of Zn/d, Zn1000 = 772 mg of Zn/d.

[2]Ewes were managed on meadow hay regrowth (d 0–42) with meadow brome (*Bromus riparius*), timothy (*Phleum pretense*), and creeping foxtail (*Alopecurus arundinaceus*) as the predominant grass species. From d 42 (≈d 133 of gestation) to parturition ewes were managed in a 40 × 40 m housed facility where they were offered *ab libitum* access to meadow hay.

Colostrum Fatty Acid Composition. Colostrum fatty acid composition was determined by GLC. Frozen colostrum samples were thawed, weighed, and freeze-dried (Labconco Freeze Dryer 5, model 75070). For each analysis, 1 mL of a 15 mg/mL internal standard solution of 13:0 (glyceryl-tritridecanoate, Sigma Chemical Co.) in chloroform was dried under nitrogen, and a 100-mg sample of freeze-dried colostrum was added. Fatty acid methyl esters were prepared using direct transesterification with an alkaline catalyst as described by Murrieta et al. (2003). Ewe colostrum conjugated linoleic acid content was quantified by gas chromatography (GC), and all samples were analyzed in duplicate according to methods described by Moutsioulis et al. (2008). Fatty acid methyl esters were separated using an Agilent 6890 GC (Agilent Technologies Inc.) equipped with a 100 mm × 0.25 mm fused silica capillary column (SP-2560, 0.2 μm film thickness, Supelco) and flame ionization detector. Fatty acid methyl ester peaks were identified by comparing retention times with fatty acid methyl esters standards (Nu-Check Prep Inc. and Matreya Inc.). Fatty acid methyl esters were evaluated using ChemStation software (Agilent Technologies, Inc.).

Statistical Analyses

Colostrum fatty acid concentrations (n = 42) were analyzed in the MIXED procedure of SAS (v9.4; SAS Institute Inc.) with fixed effects of treatment (CON, Zn500, or Zn1000), breed type (BF or WF), litter size (1 or 2+), and all 2-way interactions. Milk SCC were $\log_{10}$ transformed (logSCC) and then analyzed as repeated measures with all fixed effects described above in addition to day of lactation (d 1, ≈30, or ≈90) and all 2-way interactions. A compound symmetric covariance structure was determined to be the most parsimonious by Akaike's Information Criterion.

Binomial proportions and associated 95% confidence intervals of microbial species were analyzed within treatment and stage of lactation using the *binom* package of R (Dorai-Raj, 2014; RStudio Team, 2021). Culture- and PCR-status for each microbial species were modeled as binary variables (0 = negative, 1 = positive) in the GLIMMIX procedure and analyzed with fixed effects of treatment, breed type, and day of lactation (d ≈30 or ≈90) and the random effect of ewe. To ensure model convergence, 2-way interaction terms were removed if no statistical tendency was detected ($P > 0.10$).

RESULTS

Ten ewes were removed from the analyses because >15% of their cumulative supplement intake came from another treatment (CON = 6; Zn500 = 3; Zn1000 = 3) and 17 ewes were removed because they were not pregnant (CON = 8; Zn500 = 5; Zn1000 = 4). In total, data from 13, 21, and 17 CON, Zn500, and Zn1000 ewes remained for analyses, respectively.

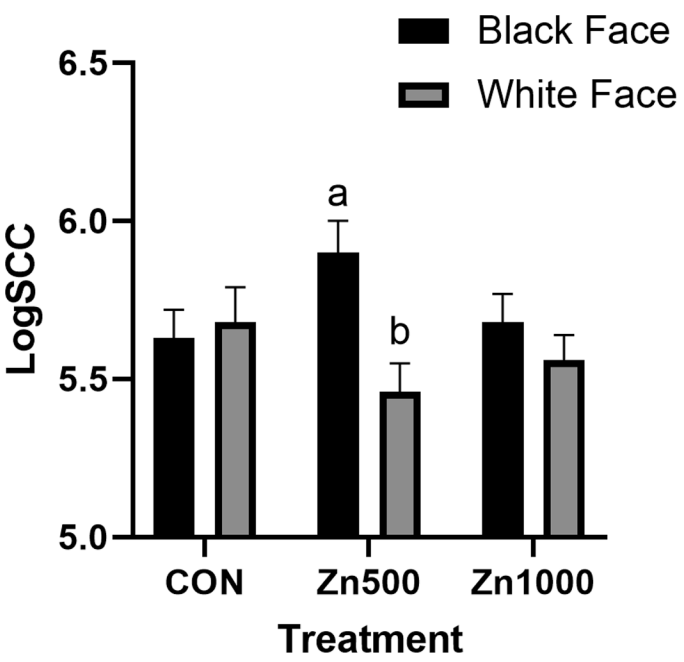

Figure 1. Least squares means for the interactive effect of treatment and breed type on $\log_{10}$ transformed SCC (LogSCC). CON = 104 mg of Zn/d; Zn500 = 462 mg of Zn/d; Zn1000 = 772 mg of Zn/d; Black Face = purebred and crossbred blackface breeds; White Face = purebred and crossbred white-face breeds. [a,b]Means within a treatment with no common letter are different ($P < 0.01$). The error bars indicate SE.

SCC

There was a treatment × breed type interaction for logSCC ($P = 0.01$) where BF had greater logSCC than WF within Zn500 (5.90 ± 0.08 vs. 5.46 ± 0.08; $P < 0.01$), but breed types did not differ within CON and Zn1000 treatments ($P \geq 0.92$; Figure 1). Least squares means for the main effects of ewe liter size and lactation period on logSCC are presented in Table 2. Ewe logSCC was greatest at weaning (6.03 ± 0.06), intermediate shortly after parturition (5.72 ± 0.06), and least at mid-lactation (5.21 ± 0.06; $P \leq 0.01$). There was no difference in logSCC between ewes bearing singles or multiples ($P = 0.57$).

Culture and PCR Microbial Results

Binomial proportions of microbial taxa identified via culture-based isolation and MALDI-TOF MS confirmation occurring at an overall proportion ≥5% within and across treatment group are presented in Tables 3 and 4. During early lactation, 77% (n = 23/30) of milk samples were positive by culture, and the most commonly identified bacteria were *Bacillus altitudinis* (35%), *Bacillus amyloliquefaciens* (26%), *Bacillus licheniformis* (30%), *Bacillus subtilis* (9%), *Staphylococcus auricularis* (17%), *Staphylococcus epidermidis* (13%), and *Staphylococcus lugdunensis* (9%). At weaning, fewer milk samples were culture positive (47%, n = 14/30), but a greater number of species were confirmed by MALDI-TOF MS at frequencies ≥5%. These microorganisms included *Achromobacter*

xylosoxidans (7%), *B. altitudinis* (43%), *B. amyloliquefaciens* (14%), *B. licheniformis* (29%), *Bacillus pumilus* (7%), *Cryptococcus neoformans* (a fungus, 7%), *Clostridium tertium* (7%), *Enterobacter pulveris* (7%), *Legionella pneumophila* (7%), *Pantoea ananatis* (7%), *Psychrobacter immobilis* (14%), *Staph. aureus* (7%), *Staph. auricularis* (14%) and *Staph. epidermidis* (7%; Table 4). The main effects of ewe breed type and treatment were not significant in the analysis of culture-status ($P > 0.51$). However, culture positivity was greater in early lactation than at weaning (77 vs. 47%, respectively; $P = 0.02$).

Overall, sample positivity determined via PCR was considerably lower than observed for culture and MALDI-TOF MS. A total of 5 and 3 of the 16 microorganisms included in the panel were detected in early lactation (Table 5) and at weaning (Table 6), respectively. During early lactation, 17% (n = 5/30) of the milk samples contained at least one of the panel targets, including *T. pyogenes* or *P. indolicus* (40%), *Staph. aureus* (20%), *Staphylococcus* spp. (60%), yeasts (20%), and the β-lactamase penicillin resistance gene in staphylococci (20%). At weaning, only 2 (7%) of the milk samples were PCR positive and included *Staph. aureus* (50%), *Staphylococcus* spp. (100%), and the β-lactamase penicillin resistance gene in staphylococci (50%). Ewe breed type, treatment, and day of lactation did not affect PCR status ($P > 0.19$).

Table 2. Least squares means (± SE) for the main effects of ewe treatment, litter size, and lactation period on $\log_{10}$ transformed SCC (logSCC; n = 51)

Effect	Level[1]	logSCC
Treatment	CON	5.65 ± 0.07
	Zn500	5.68 ± 0.05
	Zn1000	5.62 ± 0.06
Breed type	BF	5.74 ± 0.05
	WF	5.57 ± 0.05
No. of lambs born	1	5.67 ± 0.07
	2+	5.63 ± 0.04
Lactation period, d	Early (d 1)	5.72 ± 0.06[b]
	Mid-lactation (d 30)	5.21 ± 0.06[c]
	Weaning (d ≈90)	6.03 ± 0.06[a]

[a–c]Means within a column and effect with no common superscript are different ($P < 0.01$).

[1]CON = 104 mg of Zn/d, Zn500 = 462 mg of Zn/d, Zn1000 = 772 mg of Zn/d; BF = purebred and crossbred blackface breeds, WF = purebred and crossbred white-face breeds; 1 = single bearing, 2+ = multiple bearing.

Colostrum Fatty Acid Composition

Least squares means for model main effects on colostrum fatty acid concentrations are displayed in Table 7. There was no effect of treatment on any colostrum fatty acid concentration ($P \geq 0.21$). However, BF ewes had greater concentrations of palmitoleic acid (C16:1; 1.78 vs. 1.39 mg of fatty acid/100 mg of total fatty acids), heptadecenoic acid (C17:1; 0.86 vs. 0.76 mg of fatty acid/100 mg of total fatty acids), and arachidonic acid (C20:4; 0.28 vs. 0.24 mg of fatty acid/100 mg of total fatty acids; $P \leq 0.04$) compared with WF ewes.

Table 3. Number of samples collected in early lactation (18–24 h postpartum) within and across treatment and estimated species frequency (95% CI in brackets) within culture-positive samples determined by culture or matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) methods

	Treatment[1]			
Item	CON	Zn500	Zn1000	Overall
No. of samples	10	9	11	30
No. positive (%)	7 (70)	7 (77)	9 (82)	23 (77)
Species[2]				
Unidentified[3]	—	—	0.22 [0.03, 0.60]	0.09 [0.01, 0.28]
Bacillus altitudinis	0.14 [0.00, 0.58]	0.57 [0.18, 0.90]	0.33 [0.07, 0.70]	0.35 [0.16, 0.57]
Bacillus amyloliquefaciens	0.57 [0.18, 0.90]	0.29 [0.04, 0.71]	—	0.26 [0.10, 0.48]
Bacillus licheniformis	0.43 [0.10, 0.82]	0.14 [0.00, 0.58]	0.33 [0.07, 0.70]	0.30 [0.13, 0.53]
Bacillus subtilis	0.29 [0.04, 0.71]	—	—	0.09 [0.01, 0.28]
Staphylococcus auricularis	0.43 [0.10, 0.82]	—	0.11 [0.00, 0.48]	0.17 [0.05, 0.39]
Staphylococcus epidermidis	—	0.14 [0.00, 0.58]	0.22 [0.03, 0.60]	0.13 [0.03, 0.34]
Staphylococcus lugdunensis	0.14 [0.00, 0.58]	—	0.11 [0.00, 0.48]	0.09 [0.01, 0.28]

[1]CON = 104 mg of Zn/d, Zn500 = 462 mg of Zn/d, Zn1000 = 772 mg of Zn/d.

[2]Only species that occurred at an overall frequency ≥0.05 are presented.

[3]Includes samples that were culture positive but not identified by MALDI-TOF MS.

Table 4. Number of samples collected at weaning (≈90 d postpartum) within and across treatment and estimated species frequency (95% CI in brackets) within culture-positive samples determined by culture or matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) methods

	Treatment[1]			
Item	**CON**	**Zn500**	**Zn1000**	**Overall**
No. of samples	10	9	11	30
No. positive (%)	4 (40)	3 (33)	7 (64)	14 (47)
Species[2]				
Unidentified[3]	—	—	0.29 [0.04, 0.71]	0.14 [0.02, 0.43]
Achromobacter xylosoxidans	—	0.33 [0.01, 0.91]	—	0.07 [0.00, 0.34]
Bacillus altitudinis	0.25 [0.01, 0.81]	0.33 [0.01, 0.91]	0.57 [0.18, 0.90]	0.43 [0.18, 0.71]
Bacillus amyloliquefaciens	—	0.33 [0.01, 0.91]	0.14 [0.00, 0.58]	0.14 [0.02, 0.43]
Bacillus licheniformis	0.50 [0.07, 0.93]	0.33 [0.01, 0.91]	0.14 [0.00, 0.58]	0.29 [0.08, 0.58]
Bacillus pumilus	—	—	0.14 [0.00, 0.58]	0.07 [0.00, 0.34]
Cryptococcus neoformans	0.25 [0.01, 0.81]	—	—	0.07 [0.00, 0.34]
Clostridium tertium	—	—	0.14 [0.00, 0.58]	0.07 [0.00, 0.34]
Enterobacter pulveris	0.25 [0.01, 0.81]	—	—	0.07 [0.00, 0.34]
Legionella pneumophila	—	—	0.14 [0.00, 0.58]	0.07 [0.00, 0.34]
Pantoea ananatis	0.25 [0.01, 0.81]	—	—	0.07 [0.00, 0.34]
Psychrobacter immobilis	0.25 [0.01, 0.81]	0.33 [0.01, 0.91]	—	0.14 [0.02, 0.43]
Staphylococcus aureus	—	—	0.14 [0.00, 0.58]	0.07 [0.00, 0.34]
Staphylococcus auricularis	0.25 [0.01, 0.81]	—	0.14 [0.00, 0.58]	0.14 [0.02, 0.43]
Staphylococcus epidermidis	0.25 [0.01, 0.81]	—	—	0.07 [0.00, 0.34]

[1]CON = 104 mg of Zn/d, Zn500 = 462 mg of Zn/d, Zn1000 = 772 mg of Zn/d.

[2]Only species that occurred at an overall frequency ≥0.05 are presented.

[3]Includes samples that were culture positive but not identified by MALDI-TOF MS.

DISCUSSION

Zinc is key in maintaining skin integrity and mammary tissue through its role in cellular division and protein synthesis (Suttle, 2010). Specific to mammary health, Zn plays a role in keratin synthesis in the teat canal, providing protection against bacterial infection with regeneration in between milking and nursing events and may be one mechanism by which Zn decreases mastitis susceptibility (Capuco, et al., 1990; Spain, 1994; Suttle, 2010). The beneficial effects of Zn on udder health have been well documented in dairy cattle (Wedekind et al., 1992; Spain,

Table 5. Number of samples collected in early lactation (18–24 h postpartum) within and across treatment and estimated species frequency (95% CI in brackets) determined by PCR methods

	Treatment[1]			
Item	**CON**	**Zn500**	**Zn1000**	**Overall**
No. of samples	10	9	11	30
No. positive (%)	1 (10)	1 (11)	3 (27)	5 (17)
Test screen item[2]				
Trueperella pyogenes or *Peptoniphilus indolicus*	—	1.00 [0.03, 1.00]	0.33 [0.01, 0.91]	0.40 [0.05, 0.85]
β-Lactamase gene	—	—	0.33 [0.01, 0.91]	0.20 [0.01, 0.72]
Staphylococcus aureus	1.00 [0.03, 1.00]	—	—	0.20 [0.01, 0.72]
Staphylococcus spp.	—	—	1.00 [0.29, 1.00]	0.60 [0.15, 0.95]
Yeasts	—	—	0.33 [0.01, 0.91]	0.20 [0.01, 0.72]

[1]CON = 104 mg of Zn/d, Zn500 = 462 mg of Zn/d, Zn1000 = 772 mg of Zn/d.

[2]All identifications are presented.

Table 6. Number of samples collected at weaning (≈90 d postpartum) within and across and treatment and estimated species frequency (95% CI in brackets) determined by PCR methods

	Treatment[1]			
Item	**CON**	**Zn500**	**Zn1000**	**Overall**
No. of samples	10	9	11	30
No. positive (%)	1 (10)	0 (0)	1 (9)	2 (7)
Test screen item[2]				
β-Lactamase gene	—	—	1.00 [0.03, 1.00]	0.50 [0.01, 0.99]
Staphylococcus aureus	—	—	1.00 [0.03, 1.00]	0.50 [0.01, 0.99]
Staphylococcus spp.	1.00 [0.03, 1.00]	—	1.00 [0.03, 1.00]	1.00 [0.16, 1.00]

[1]CON = 104 mg of Zn/d, Zn500 = 462 mg of Zn/d, Zn1000 = 772 mg of Zn/d.

[2]All identifications are presented.

1994; Kellogg et al., 2004; Pechová et al., 2006; Cope et al., 2009). Similarly, Saianda et al. (2007) reported a significant reduction in bacterial adherence to the mammary epithelium in dairy ewes supplemented with 30 mg/kg Zn DM compared with unsupplemented ewes. Greater research emphasis has been placed on Zn deficiency compared with feeding dietary Zn more than recommended levels (1, 4, and 7 × NASEM) such as those in the current study. Cope et al. (2009) observed a decrease in dairy cow milk SCC when they were fed either an inorganic (ZnO) or organic form (chelated) of Zn at recommended dietary concentrations compared with approximately 66% of the daily recommendation. In contrast, Pechová et al. (2006) fed dairy cattle ≈4 times the recommended dietary Zn concentration, and although authors observed no difference in milk Zn concentrations, they reported a reduction in SCC by 45% in mo 3 of dietary treatment during lactation. Adequacy of Zn status pre- and postpartum has been associated with a decrease in SCC in dairy cattle and sheep (Davidov et al., 2013; Murphy et al., 2018). Although treatments were not applied into lactation and Zn concentrations of colostrum and milk were not quantified in the current study, successful Zn fortification of the milk up to 30 d into lactation has been observed when

Table 7. Least squares means (± SE) for the main effects of treatment and ewe breed type on milk fatty acid concentration (mg/100 mg of fatty acids) at 12 to 18 h after parturition (n = 47)

	Treatment[1]			Breed type[2]	
Fatty acid	**CON**	**Zn500**	**Zn1000**	**BF**	**WF**
C8:0	0.63 ± 0.07	0.56 ± 0.06	0.62 ± 0.07	0.63 ± 0.05	0.59 ± 0.05
C10:0	2.42 ± 0.29	2.16 ± 0.24	2.40 ± 0.27	2.42 ± 0.21	2.26 ± 0.22
C12:0	2.25 ± 0.15	2.08 ± 0.13	2.32 ± 0.14	2.18 ± 0.11	2.26 ± 0.12
C14:0	9.94 ± 0.44	9.14 ± 0.36	9.76 ± 0.41	9.43 ± 0.33	9.80 ± 0.34
C15:0	0.95 ± 0.08	0.96 ± 0.06	0.93 ± 0.07	0.97 ± 0.06	0.92 ± 0.06
C16:0	29.40 ± 0.96	27.96 ± 0.79	28.19 ± 0.89	28.74 ± 0.71	28.29 ± 0.74
C16:1	1.62 ± 0.09	1.51 ± 0.07	1.63 ± 0.08	1.78 ± 0.07[a]	1.39 ± 0.07[b]
C17:0	1.14 ± 0.04	1.22 ± 0.03	1.18 ± 0.03	1.18 ± 0.03	1.18 ± 0.03
C17:1	0.80 ± 0.04	0.82 ± 0.03	0.83 ± 0.04	0.86 ± 0.03[a]	0.78 ± 0.03[b]
C18:0	6.67 ± 0.42	7.36 ± 0.35	6.88 ± 0.39	6.70 ± 0.31	7.24 ± 0.32
C18:1	34.70 ± 1.55	36.62 ± 1.28	35.76 ± 1.44	35.28 ± 1.15	36.11 ± 1.19
C18:2	2.76 ± 0.07	2.89 ± 0.05	2.77 ± 0.06	2.77 ± 0.05	2.85 ± 0.05
C18:2 *cis*-9,*trans*-11	1.00 ± 0.04	0.99 ± 0.04	1.04 ± 0.04	1.00 ± 0.03	1.02 ± 0.03
C18:3	1.23 ± 0.06	1.18 ± 0.05	1.23 ± 0.05	1.22 ± 0.04	1.22 ± 0.04
C20:4	0.26 ± 0.02	0.25 ± 0.01	0.26 ± 0.02	0.28 ± 0.01[a]	0.23 ± 0.01[b]

[a,b]Means within an effect with no common superscript are different ($P < 0.01$).

[1]CON = 104 mg of Zn/d, Zn500 = 462 mg of Zn/d, Zn1000 = 772 mg of Zn/d.

[2]BF = purebred and crossbred black-face breeds, WF = purebred and crossbred white-face breeds.

feeding $ZnSO_4$ to meat-type ewes ≈1 and ≈4 times NASEM recommended levels (Page et al., 2020). Zinc requirements increase ≈41% from breeding to late gestation but only ≈5% from late gestation to early lactation (NASEM, 2007), which are unlikely to be met in semi-extensive sheep production systems of the western United States.

Results from the present study found a significant treatment × breed type effect on logSCC. When fed additional dietary Zn concentrations (≈4 times NASEM), BF ewes had greater logSCC than WF ewes, an effect not observed when dietary Zn levels were fed at ≈1 and 7 times NASEM recommended concentrations. This finding points to the importance of understanding the production factors that contribute to how much dietary Zn is required (BW, gain, fetal weight, milk production, and wool) and how requirements may differ based on breed type. Snowder and Glimp (1991) observed 13 to 17% greater milk production from Suffolk-type ewes compared with Rambouillet, Polypay, and Columbia ewes, which would translate to greater Zn demands when extrapolated to the lactational Zn demands in BF type ewes in the current study. In part 1 of this study (Stewart et al., 2020), BF and WF ewes did not differ in serum Zn concentrations at the time of lambing, but WF lambs had ≈28% greater serum Zn concentration and 60% greater calculated transfer efficiency than BF lambs. Our study observed increases in SCC and microbial positivity rates based on the day of lactation, with larger increases during early lactation in comparison with mid-lactation, and weaning had the greatest SCC. This may not be surprising as Huntley et al. (2012) and Paape et al. (2007) both reported a decrease in ewe SCC in the second month of lactation, hypothesizing that increased milk production caused the decrease by dilution.

The combination of SCC and microbiological analyses from milk in the current study provide a more holistic investigation into the effects of dietary Zn on ewe mammary health and immune response. Bacteria were commonly isolated from milk samples collected during early lactation (d 1; 77%) and weaning (≈90 d; 47%), and the most commonly identified isolates included *Bacillus* spp. and *Staphylococcus* spp., coinciding with other mastitis research in sheep (Zadoks et al., 2014; Knuth et al., 2021; Van den Crommenacker-Konings et al., 2021). Though bacilli are commonly present in soil, both culture-dependent (Watkins et al., 1991; Al-Majali and Jawabreh, 2003; Arsenault et al., 2008; Spanu et al., 2011) and culture-independent (Bhatt et al., 2012; Braem et al., 2013; Bonsaglia et al., 2017) techniques have identified the genus as a member of the milk and teat microbial communities. A clear link between *Bacillus* spp. and ovine milk has been established (Fotou et al., 2011). *Staphylococcus aureus* are commonly found on the skin and in the environment and have been isolated and identified as a major mastitis-causing pathogen in cases of ovine subclinical mastitis (Watson et al., 1990; Arsenault et al., 2008). Coagulase-negative staphylococci such as *Staph. epidermidis* and *Staph. lugdunensis* are also common members of the skin and mucus membrane microbial communities. Furthermore, both have been previously isolated in cases of ovine subclinical mastitis and are a group of mastitis-causing pathogens more common in nondairy systems (Arsenault et al., 2008). Together, these studies illustrate the pathogenic-nature of bacilli and staphylococci in relationship to mammary health. This has notable repercussions, because certain bacterial species including *E. coli*, *Staph. aureus*, and streptococci typically cause clinical infections, whereas the majority of mastitis cases present in a subclinical state with no visible signs and can be the result of infection from numerous bacteria species (i.e., *Staphylococcus* spp., including *Staph. aureus*, and CNS; Rovai et al., 2014). In the present study, samples were first collected at 12 to 18 h after parturition, so there may be some remaining colostrum properties compared with milk collected at weaning. The inherent differences (e.g., fat and protein content, mineral concentrations, enzyme activity, and so on) discernable between milk and colostrum composition could contribute to the nutrient availability supporting growth of mastitis-related microorganisms, therefore partially accounting for the differences observed in subclinical mastitis prevalence at these time points. Culture-based methods were not always on concordance with the results of molecular detection. This is to be expected, because multiple microbial species can be isolated and identified using cultural isolation and mass spectrometry–based identification, but PCR is limited to detecting targets that are part of the testing panel. The ability of PCR to detect nonviable organisms (Taponen et al., 2009), and the perceived increased sensitivity compared with culture (Cederlöf et al., 2012; Keane et al., 2013), partially accounts for such differences. However, PCR results must be used cautiously, because interpretation may be complicated when more than one target species is present in samples or false-positive results are recorded in absence of an inflammatory response (Koskinen et al., 2010).

Mild Zn deficiency during the periods of late gestation and early lactation may impair immune function as Zn is a key nutrient in both innate and adaptive immunity (Ibs and Rink, 2003; Zhou et al., 2021). Furthermore, Zn can exhibit antibacterial activity (Etaiw et al., 2011) and may help maintain epithelial cell integrity (Saianda et al., 2007). Together, these properties suggest that dietary Zn supplementation could serve as a tool to control etiological agents of mastitis. However, the present study fell short of supporting a link between the status of culture or mastitis status and increasing dietary Zn concentrations. Starke et al. (2013) identified pronounced reductions of *Enterobacteriaceae*, *E. coli*, and *Lactobacillus* in the intestines of weaned piglets supplemented with zinc oxide, though duration of the reduction was dependent on the bacterium. Because Zn has antibacterial properties, researchers have investigated the clinical mastitis resistome and have revealed Zn-resistant *Shigella* and *Enterobacter* spp. (Hoque

et al., 2019). This raises concern that even if dietary zinc supplementation could reduce the prevalence and effects of subclinical mastitis, Zn-resistant bacterial populations may dampen the benefits of supranutritional Zn supplementation. Despite whether the beneficial effects of Zn reside in its nutraceutical or antibacterial properties, indices of mammary health were unaltered in the current study.

Others have evaluated effects of differing dietary Zn concentrations on milk fatty acid production in dairy cattle. Wiking et al. (2008) reported that milk production and fatty acid composition were unaffected by dietary Zn treatment (39 vs. 83 mg of Zn/kg of DM). Hermansen et al. (1995) also observed no change in free fatty acids (**FFA**) between dietary Zn concentrations immediately after milking, although Zn treatment (90 mg of Zn/kg of DM) decreased FFA compared with control (34 mg of Zn/kg of DM) cows after 72 h of chilling. In the present study there was no Zn treatment effect on milk fatty acid concentrations. However, interesting breed effects were observed as BF ewes had greater concentrations of palmitoleic, heptadecenoic, and arachidonic acids than WF ewes. White-face ewes had numerically greater amounts of linoleic acid, the precursor for arachidonic acid, which was 22% greater in BF ewes. Breed differences in de novo fatty acid synthesis could explain this difference with BF ewes converting a greater percentage of linoleic acid to arachidonic acid. Although few studies have focused on milk FFA differences between breeds, Signorelli et al. (2008) reported differences in milk FFA composition including heptadecenoic acid (C16:1) between 3 native Italian sheep breeds. These breed differences in FFA milk composition may also contribute modestly to preweaning growth differences of BF versus WF lambs. Overall fatty acid composition in the current study is similar to concentrations reported by Casoli et al. (1989) with palmitic, oleic, stearic, and myristic acids making up the highest percentages in the fatty acid profile of Massese ewes. In the current study, colostrum fatty acid composition was only measured at lambing. However, future research may look at the effects of Zn fed throughout lactation on temporal changes in FFA composition, as others have reported changes in ewe milk fatty acid composition throughout lactation (Casoli et al., 1989; Nudda et al., 2005).

In conclusion, supplementing primiparous ewes with $ZnSO_4$ during late gestation to increase overall dietary Zn intake up to 7-fold the current NASEM recommendations did not affect SCC, positivity rates of mastitis-related microorganisms, or colostrum fatty acid profiles. Nevertheless, the observed elevated SCC and the established frequency of positive culture and PCR-positive samples indicate the need for preventative mastitis interventions in nondairy sheep, particularly around the time of parturition. Differential biological responses to Zn treatment by breed type in part 1 of this research and the current SCC data may indicate a differing triage of Zn utilization for production parameters (i.e., milk production, BW, wool growth, and so on; Stewart et al., 2020).

APPLICATIONS

Including $ZnSO_4$ above NASEM recommendations during late gestation had little effect on altering milk SCC and colostrum fatty acid profile of semi-extensively managed, primiparous ewes. However, positive microbial culture results suggest subclinical mastitis to be more prevalent during early lactation than at weaning, and etiological agents include bacilli and staphylococci. Therefore, future research is warranted to investigate additional management methods that reduce bacterial load and target mechanisms by which pathogenic bacteria infect the mammary gland. Vast breed diversity and specialized sheep production systems internationally may benefit from precision mineral supplementation programs. Breed-type differences in SCC when fed Zn at ≈4 × NASEM concentrations indicate the nuance of breed and related production levels when implementing mineral supplementation strategies for pregnant ewes.

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