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RESEARCH ARTICLE

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Association between milk anti-*Staphylococcus aureus* antibody, somatic cell count, and milk yield in lactating dairy cows

S McDougall[a,b], R Koralagamage Don[c]* and A Pernthaner[c]

[a]Tāwharau Ora – School of Veterinary Science, Massey University, Palmerston North, New Zealand; [b]Cognosco, Anexa Veterinary Services, Morrinsville, New Zealand; [c]Koru Biotech Solutions Ltd, Palmerston North, New Zealand

ABSTRACT

Aims: To assess the association between the sample/positive (S/P) ratios from a milk IgG ELISA, specific for *Staphylococcus aureus* and the somatic cell count (SCC) in cow composite milk samples taken at routine herd testing, and the association between the S/P ratio and milk solids (MS; kg fat + protein/cow/day) in cows in a pasture-based, seasonal-calving, New Zealand dairy herd.

Methods: Data from cow-composite milk samples were retrospectively analysed to determine the associations between the S/P ratios from a milk IgG ELISA specific for *S. aureus*, SCC (transformed to linear scores (LS) for analysis), and MS from cow-composite milk samples collected on four occasions across two lactations. A generalised linear mixed model was used to assess the effect of age, breed, month in lactation, and their interactions on the S/P ratio. To assess agreement between cows defined as infected based on S/P and on SCC, cows were classified as uninfected or infected using test positive cutpoints $\geq$ 0.38 for the S/P ratio and $\geq$ 150,000 cells/mL for SCC, and the agreement between these categories was compared by Gwet's agreement coefficient (AC). A generalised linear mixed model was used to assess associations between S/P and MS production with age, breed, and herd test date, with their interactions as explanatory variables, and cow as a random effect.

Results: The S/P varied by month of lactation, and there was an interaction between age and breed. There was a moderate agreement between the dichotomised S/P ratios and the dichotomised SCC (Gwet's AC = 0.59 (SE = 0.02); $p < 0.001$). When the MS production of cows defined as S/P test negative, suspicious or positive was analysed, there was an age by S/P interaction whereby there was no association between S/P category and MS amongst 2-year-olds, while in older cows, test-positive cows had lower MS than test-negative cows.

Conclusions: This observational study demonstrated moderate agreement between S/P ratio and SCC. Additionally, there were negative associations between the S/P ratio and MS amongst older cows and in late lactation. Thus, the S/P ratio of an *S. aureus*-specific IgG ELISA is correlated with a well-defined marker of mastitis (SCC) and with milk yield depression. Further assessment of these associations using microbiology, and investigation of associations between the antibody results and long-term survival in the herd would further strengthen the validity and utility of the test.

Abbreviations: AC: Agreement coefficient; AIC: Akaike information criteria; BIC: Bayesian information criteria; IMI: Intramammary infection; LS: Linear score; MS: Milk solids (i.e kg milk fat + protein/cow/day); SCC: Somatic cell count; S/P: Ratio of sample to positive optical density in the *Staphylococcus aureus*-specific ELISA

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KEYWORDS
Dairy cow; mastitis; *Staphylococcus aureus*; somatic cell count; milk yield

Introduction

Inflammation of the mammary gland (mastitis) is one of the most common bovine diseases (Ruegg 2017) and has a substantial economic impact (Hogeveen *et al*. 2011). *Staphylococcus aureus* is a common cause of mastitis, with many *S. aureus* cases being subclinical – characterised by elevated somatic cell counts (SCC) in the absence of grossly visible signs of inflammation.

Due to intermittent shedding of *S. aureus* into milk, diagnosis of *S. aureus* mastitis is challenging. The sensitivity of bacterial culture for detection of *S. aureus* in a single quarter milk sample is about 90% (Sears *et al*. 1990; Dohoo *et al*. 2011). Sensitivity can be increased when samples are interpreted in parallel, but this increases the cost of diagnosis (Zecconi 2010). Indirect tests for intramammary infection (IMI), such as elevated counts of white blood cells in milk (e.g. SCC), are widely used as a proxy for IMI, enabling monitoring of the prevalence and incidence of new IMI. The relatively lower cost and wide availability of SCC encourage its use relative to more costly microbiology (Schukken *et al*. 2003). The sensitivity and specificity

CONTACT S McDougall smcdouga@massey.ac.nz
*Current address: Fonterra Co-operative Group Limited, Palmerston North, New Zealand

in detecting an IMI associated with any pathogen using SCC were 89% and 75%, respectively, at a cut point of 200,000 cells/mL (McDermott *et al*. 1982). The sensitivity and specificity of cow-composite SCC specifically for *S. aureus* were reported to be 54% and 83%, respectively, using a cut point of 250,000 cells/mL (Buelow *et al*. 1996). An increase in cow-composite SCC from $< 200{,}000$ to $\geq 200{,}000$ cells/mL between sequential herd tests has been shown to have a sensitivity and specificity for detection of new IMI of 72% and 86%, respectively (Dohoo and Leslie 1991). Due to the non-normal distribution of SCC and a non-linear relationship between SCC and milk yield, the linear score (LS; $3 \times \log_2 (SCC/100{,}000 + 3)$) (Wiggans and Shook 1987) has been used to assess associations between SCC and milk yield, as LS demonstrates a linear relationship with milk yield loss, with each doubling of LS associated with approximately 2% milk yield loss (Jones *et al*. 1984). However, SCC is a marker of inflammation rather than infection and is not pathogen-specific; hence it will always have limits in terms of sensitivity and specificity for IMI (Wiggans and Shook 1987; Schukken *et al*. 2003). Novel diagnostic tests with high diagnostic sensitivity that are pathogen-specific and that are simple to use, are thus required to improve mastitis control programmes. Use of non-culture-based tests such as a milk antibody ELISA may provide an alternative diagnostic approach.

The production of *S. aureus*-specific antibodies is an essential part of the host-specific immune response to infection. Several studies report use of immunoassays in the detection of *S. aureus*-specific antibodies to determine infection (Grove and Jones 1992; Yazdankhah *et al*. 1998; Fox and Adams 2000). *S. aureus*-specific IgG in milk are detected by approximately 2 weeks post-challenge (Fox and Adams 2000), suggesting that antibody-based detection is more suitable for the identification of persistent rather than acute, short-duration infections. Leitner *et al*. (2000) demonstrated an association between chronic *S. aureus* IMI (as defined by a history of *S. aureus* infection in mid-lactation in multiparous cows) and a specific IgG response in serum and milk, indicating that antigen-specific IgG is a suitable biomarker for infection. The sensitivity and specificity of two different IgG-specific antibody ELISA were 83%–90% and 97–100%, respectively (Grove and Jones 1992; Leitner *et al*. 2000). There was a clear separation in cow-composite *S. aureus* milk antibody titres at the beginning of the subsequent lactation amongst those cows remaining infected (culture-positive at one or more milk samples collected at 1, 4, 7 and 11 days post-calving) and those cured (culture-negative at each of these time points) following pre-dry-off challenge with *S. aureus* and subsequent treatment with antimicrobial dry cow therapy. This indicates that specific antibody titres do not persist in the next lactation in cows cured during the dry period (Fox and Adams 2000). The titres of one *S. aureus*-specific milk IgG ELISA (ProStaph 1; Proscience Corporation, Sterling, VA, USA) were quadratically associated with increasing SCC (Grove and Jones 1992). More recently, an ELISA for the detection of *S. aureus*-specific IgG in bovine milk (StaphGold; Koru Diagnostics, Palmerston North, NZ) has been commercialised (Yang and Laven 2022). This test was found to have a sensitivity and specificity of 90% and 95%, respectively, as determined by a Gaussian mixture model, and is the test used in the current study.

Intramammary infection with *S. aureus,* defined by conventional microbiology, is associated with reduced milk yield in subclinical (Botaro *et al*. 2015) and clinical (Gröhn *et al*. 2004) cases. Hence, we hypothesise that historical or current infection associated with *S. aureus,* as determined by elevated *S. aureus* antibodies, would be associated with reduced milk yield.

The objectives of this retrospective observational study were to assess in cow-composite milk samples the associations between the S/P ratio of an *S. aureus*-specific IgG ELISA, SCC, and milk yield, in a pasture-based, seasonal-calving dairy herd.

Materials and methods

Study herd and sampling

This study was conducted between April 2023 and April 2024 in a herd of approximately 1,100 cows, located in the Manawatū region of New Zealand. The herd was selected on a convenience basis, as herd testing and the *S. aureus*-specific antibody ELISA testing had already been undertaken. The cows calved in spring (i.e. between 1 July and 30 September 2023) and were dried off in autumn (i.e. April, May or June 2024). Cows in their first and second lactation (approximately 40% of the herd) were predominantly crossbreeds (Holstein-Friesians crossed with Jerseys), while cows from the third lactation onwards (approximately 60%) were predominantly purebred Holstein-Friesians. Cows were managed in a predominantly pasture-based system with supplementation with palm kernel expeller to fill deficits in pasture availability. Twice-a-day milking occurred in an 80-bay rotary milking shed with manual cup removal and manual post-milking teat spraying. Mean total lactation cow production for the 2023/2024 lactation was approximately 400 kg milk solids (MS; i.e. sum of kg fat and protein/cow/lactation). Each cow was treated at the end of lactation by intramammary infusion into each quarter of 300 mg cephapirin benzathine (Cefa-Safe; Schering-Plough Animal Health Ltd., Upper Hutt, NZ) and 2.6 g of bismuth nitrate as an internal teat sealant (Teatseal, Zoetis NZ, Auckland, NZ in 2023; Shutout, MSD Animal Health NZ, Upper Hutt,

NZ in 2024). Clinical mastitis cases were treated by intramammary infusion on three occasions with 1 g penicillin (Intracillin 1000 Milking Cow; Virbac NZ Ltd., Hamilton, NZ). Of the total herd population, 5.6% were removed for udder related reasons in the 2023/24 season.

Animal ethics committee approval was not sought for the study as the milk samples used for the study had been collected as part of routine production recording (herd testing) on the farm and hence no additional animal manipulations were undertaken for the purpose of the study.

Testing frequencies

StaphGold testing was conducted on cow-composite milk samples from all cows that were present at herd tests in April 2023, September 2023, December 2023, and April 2024. Herd testing was conducted by Livestock Improvement Corporation (LIC, Hamilton, NZ); SCC data and MS data were obtained from these herd tests, and additionally in January 2024. Milk solids production and SCC were determined using validated fluoro-optic methods (CombiFoss7; FOSS, Hillerød, Denmark) in a commercial laboratory (LIC).

The herd test milk samples were preserved with bronopol. From each composite cow milk sample, an automated defatting and subsampling system was used to deposit approximately 1.5 mL of each milk sample into 96-deepwell plates that were stored frozen until testing for *S. aureus*-specific IgG.

S. aureus*-specific IgG testing*

Testing for *S. aureus*-specific IgG was conducted using a commercially available ELISA test (StaphGold) according to the instructions for users. Defatted herd test samples diluted 1:10 were assayed. Antibody data were read at a wavelength of 450 nm with 650 nm as a reference, and the results were expressed as the test sample to positive control (S/P) ratio[1].

Results were recorded on a continuous basis and subsequently categorised as antibody positive (Pos, S/P $\geq$ 0.38), suspicious positive (Sus, S/P 0.22–0.37), or negative (Neg, S/P < 0.22) as recommended by the manufacturer. The S/P data were also dichotomised as < 0.38 *vs.* $\geq$ 0.38 for some analyses. The range of S/P values was −0.27 to 2.93.

Analysis of data

Data were collated in an Excel spreadsheet (Microsoft, Redmond, WA, USA) prior to importation into a statistics package (STATA v18.1, STATA Corp, College Station, TX, USA) for analyses.

Data handling

Cow demographic data including age, calving date, breed, removal date and reasons, and treatment records were obtained from electronic records of the herd (MINDA; LIC). The MS, SCC and S/P data at each herd test were collated. Cows were defined test-positive (i.e. likely infected) if the SCC was ≥150,000 cells/mL. The *S. aureus* IgG ELISA results were classified as either test-positive or test-negative (i.e. dichotomised as test-negative where the S/P ratio < 0.38 or test-positive where the S/P ratio $\geq$ 0.38) or categorised as negative (S/P < 0.22), suspicious (S/P 0.22–0.37) or positive ($\geq$ 0.38) as per the manufacturer's recommendations. Additionally, the SCC status change was defined using sequential SCC results to categorise cows as not infected (i.e. two sequential SCC < 150,000 cells/mL), newly infected (i.e. < 150,000 cells/mL at the preceding herd test, and $\geq$ 150,000 cells/mL at the subsequent herd test), cured (i.e. $\geq$ 150,000 cells/mL at the preceding herd test and < 150,000 cells/mL at the subsequent herd test), and chronically infected (i.e. two sequential herd tests $\geq$ 150,000 cells/mL). Similarly, the StaphGold status change was defined as uninfected (i.e. two sequential tests negative), new infection (preceding test negative, current test positive), self-cure (i.e. preceding test positive, current test negative), and chronically infected (i.e. two sequential tests positive). Suspicious tests were coded as test negative for these analyses.

Analysis of the S/P ratio

A generalised linear mixed model of the S/P ratio as a continuous variable was undertaken using age (categorised into years, with cows $\geq$ 9 years of age collapsed into one category), month in milk at herd test (by month for 1–10, with those cows with a lactation >10 months collapsed into the 10-month category), and breed coded as Friesian (i.e. > 11/16^{ths} Friesian, otherwise coded as crossbred) as categorical explanatory variables. Month in lactation was used as a time variable (rather than herd test date) in these analyses due to the distribution of calving dates being such that animals varied in stage of lactation by up to 3 months at any given herd test. Univariable associations between the S/P ratio and possible explanatory variables were initially undertaken using one-way ANOVA, and those associated ($p < 0.2$) were offered to a forward stepwise manual model-building process. The Bayesian (BIC) and Akaike information criteria (AIC) were used to assess whether to add a variable to the model, with lower AIC and BIC indicative of improved model fit. Cow was treated as a random effect. First-order interactions of the remaining fixed effects were tested and included in the final model if $p < 0.05$. Estimated marginal means and 95% CI were calculated at

[1] $\text{S/P ratio} = \dfrac{\text{sample absorbance} - \text{negative control absorbance}}{\text{positive control absorbance} - \text{negative control absorbance}}$

the observed frequencies of the covariates in the model and multiple comparisons were undertaken using the Bonferroni correction. To assess the model, the likelihood ratios were calculated and tested for models with and without cow as a random effect, and standardised residuals plotted to check for normality.

A binary logistic regression model was used to assess associations between cows defined as having likely acquired a new IMI, defined as a change in S/P ratio from < 0.38 to ≥ 0.38, or having remained uninfected between sequential tests (i.e. S/P ratio < 0.38 at both tests). Explanatory variables included age, breed, and herd test date as fixed explanatory variables, and the milk solids production at the preceding herd test was used as a continuous explanatory variable. Cow was included as a random effect using an unstructured covariance matrix. For these analyses, only cows that were uninfected at the preceding time point were eligible for inclusion. The unadjusted incidence rate by breed and herd test was calculated as the exact binomial confidence interval of the number of newly infected animals divided by the sum of the newly infected and uninfected cows.

Association between S/P and SCC

Scatter plots of LS *vs.* S/P were created for each herd test. The agreement between the SCC categories (i.e. < 150,000 cells/mL *vs.* ≥ 150,000 cells/mL) and the S/P category (dichotomised as < 0.38 *vs.* ≥ 0.38) was assessed by chance-corrected agreement coefficients (i.e. Gwet's AC) incorporating both the number of categories and the frequency with which they are used, and with a probabilistic interpretation of level of agreement between tests (that is, accepting that there is uncertainty about the estimate of the level of agreement), as implemented in the STATA package kappaetc (Klein 2018). Similarly, the change in infection status based on SCC and S/P ratio categories (as defined above) were tabulated and agreement assessed. The benchmark for the level of agreement was as previously defined (Landis and Koch 1977).

Relationship between S/P and milk solids production

The MS production was modelled using two generalised linear mixed models. Explanatory variables offered to the models included age, breed, and herd test date, and with S/P test results categorised as negative, suspicious or positive (i.e. S/P ratio categorised into three levels), or as the change in status of the S/P ratio between the previous and current herd tests as defined above (i.e. as uninfected, newly infected, cured or chronically infected). Cow was included as a random effect using an unstructured covariance matrix. The improvement in model fit and for variable selection during the forward manual model building was assessed by AIC, BIC, and likelihood-ratio tests.

Results

Staphylococcus aureus-*specific IgG ratios*

In a generalised linear mixed model, the S/P ratio varied by month in milk ($p < 0.001$; Figure 1(a)) with the highest S/P ratio occurring in the first month of lactation, the lowest occurring in the second month, and increasing thereafter. S/P ratio varied by age ($p = 0.007$) and breed ($p = 0.001$), and there was a complex breed by age interaction (Figure 1(b)), as amongst the Holstein-Friesians, the S/P ratio was higher in younger than older cows, whereas for crossbred animals, the inverse was true.

The S/P defined incidence of new infections, defined as the proportion of cows with a change in the S/P ratio from < 0.38 to ≥ 0.38 between sequential

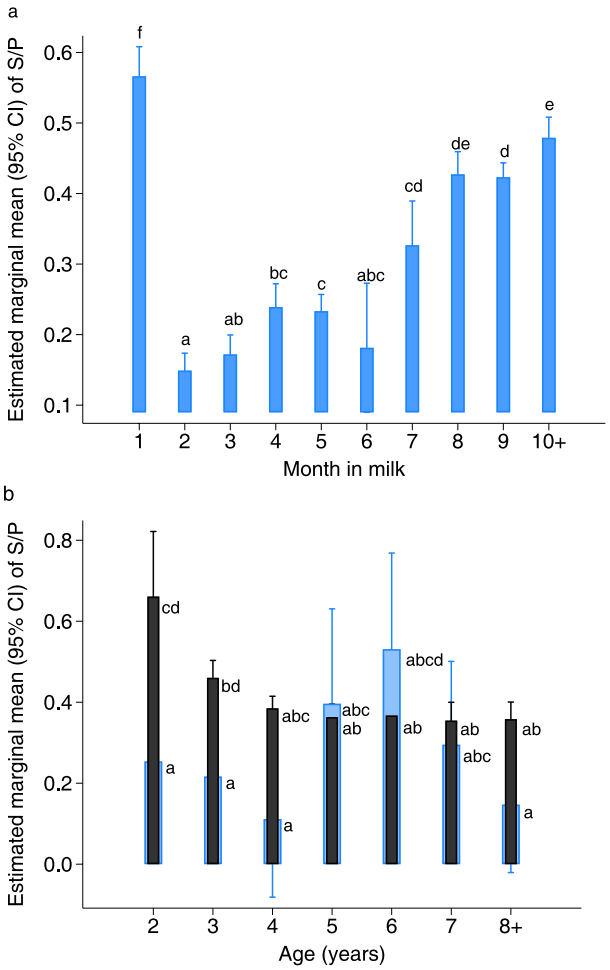

Figure 1. Estimated marginal means (95% CI) of the sample/positive (S/P) ratios from a milk IgG ELISA, specific for *Staphylococcus aureus* by (a) month of lactation, and (b) by age across the interaction with breed (Friesian = open bar; Crossbred = blue bar), from a generalised linear mixed model of factors affecting S/P ratio in a single, seasonal calving pastoral dairy herd from the Manawatū region of New Zealand. Columns with differing superscripts differ at $p < 0.05$.

Table 1. Results of a binary logistic regression analysis to assess associations between the incidence of new intramammary infection[a] and age, herd test date, and milk solids (fat plus protein) production at the preceding herd test, from a single, seasonal calving pastoral dairy herd from the Manawatū region of New Zealand where cows were sampled at herd tests carried out in April 2023, September 2023, December 2023, and April 2024.

Variable	Coefficient (95% CI)	P-value	OR (95% CI)	n/N[b]	Incidence (95% CI)[c]
Age (years)					
2	Ref.	0.000		87/461	0.19 (0.15–0.23)
3	−0.07 (−0.58 to 0.43)	0.778	0.93 (0.56–1.54)	72/480	0.15 (0.12–0.19)
≥ 4	0.93 (0.44–1.42)	0.000	2.53 (1.56–4.12)	269/1,086	0.25 (0.22–0.27)
Herd test					
Sep 2023	Ref.	0.000		78/473	0.16 (0.13–0.20)
Dec 2023	0.31 (−0.15 to 0.77)	0.189	1.36 (0.86–2.16)	143/865	0.17 (0.14–0.19)
Apr 2024	1.14 (0.71–1.56)	0.000	3.12 (2.04–4.78)	207/689	0.30 (0.27–0.34)
Milk solids (kg/cow/day)	−0.50 (−0.81 to −0.20)	0.001	0.61 (0.45–0.82)		
Intercept	−1.79 (−2.38 to −1.21)	0.000	0.00 (0.00–0.00)		

[a]Defined as a change in S/P ratio from < 0.38 to ≥ 0.38, where S/P = ratio of sample to positive optical density in the *Staphylococcus aureus*-specific ELISA.
[b]Number of newly infected cows (n) and the number of animals eligible to become infected (i.e. those uninfected at the preceding herd test (N)).
[c]Incidence between sequential herd tests (i.e. approximately 2 months, calculated as the exact binomial proportion from the n/N data).

herd tests, was higher for ≥ 4 year-old cows compared with 2- or 3-year-old cows (0.19 (95% CI = 0.15–0.23), 0.15 (95% CI = 0.12–0.198), and 0.25 (95% CI = 0.22–0.27) for 2-, 3- and ≥ 4 year-old cows, respectively, p = 0.005) and was higher late in lactation (April 2024) compared to early and mid-lactation (September 2023 and December 2023 herd tests) (p < 0.001, Table 1). The MS production at the preceding herd test was negatively associated with incidence (coefficient = −0.50; p < 0.001; OR = 0.61; Table 1), and this was consistent across age groups.

Association between SCC and S. aureus*-specific S/P ratios*

There was a positive correlation between LS and S/P ratio at each herd test (Figure 2).

For all herd tests, the unadjusted agreement was 72.5% following dichotomisation of the S/P ratios and the SCC, with a moderate chance-corrected agreement (Gwet's AC = 0.59 (95% CI 0.56–0.63); p < 0.001; Table 2). Following categorisation as uninfected, newly infected, cured or chronically infected using S/P ratio and SCC, the unadjusted agreement was 56.5%, with a moderate chance-corrected agreement (Gwet's AC = 0.46 (95% CI 0.44–0.48); p < 0.001; Table 3).

Relationship between S. aureus*-specific IgG and milk yield*

In the generalised mixed linear model with cow as a random effect, MS production varied with age and herd test date, and for cows > 2 years old, was negatively associated with S/P status when categorised as negative, suspicious or positive, (all p < 0.001; Table 4). However, there was an interaction of S/P status and age (p < 0.001) whereby among 2-year-old cows, there was no difference in MS production between the S/P status groups. Whereas there was higher MS production for test-negative than test-positive 3-year-old cows (with suspicious cows intermediate), and amongst cows ≥ 4 years old, MS production differed significantly among all three test groups (Figure 3(a)),

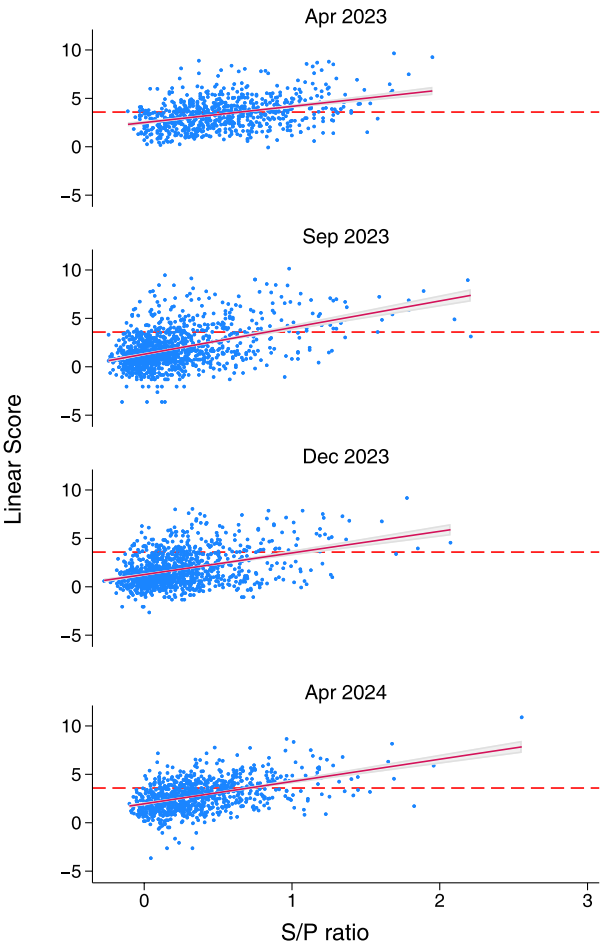

Figure 2. Scatter plots of the somatic cell count (SCC) linear score (log base 2 of SCC ÷100,000 + 3) and the ratio of sample to positive (S/P) optical density in a *Staphylococcus aureus*-specific ELISA, carried out contemporaneously on milk samples collected at four herd tests carried out between April 2023 and April 2024 in a single, seasonal calving pastoral dairy herd from the Manawatū region of New Zealand. The line of best fit is indicated by the solid red line (95% CI as the grey area), and the horizontal dashed red line indicates a SCC of 200,000 cells/mL.

Table 2. Number of test results from a single, seasonal calving pasture-fed dairy herd from the Manawatū region of New Zealand, categorised by somatic cell count (SCC; uninfected when < 150,000 cells/mL, infected when ≥ 150,000 cells/mL) and S/P[a] category (uninfected when < 0.38, infected when ≥ 0.38) recorded at herd tests carried out in April 2023, September 2023, December 2023, and April 2024.

	S/P ratio		
SCC category	< 0.38	≥ 0.38	Total
< 150,000 cells/mL	2,188	690	2,878
≥ 150,000 cells/mL	360	578	938
Total	2,548	1,268	3,816

[a]S/P: ratio of sample to positive optical density in the *Staphylococcus aureus*-specific ELISA

Table 3. Number of test results categorised by change in somatic cell count (SCC) *vs.* change in S/P[a] category recorded between sequential herd tests carried out in April 2023, September 2023, December 2023, and April 2024 for a single, seasonal calving pastoral dairy herd from the Manawatū region of New Zealand.

	S/P category[b]				
SCC category[c]	Uninfected	Newly infected	Cured	Chronically infected	Total
Uninfected	1,181	236	277	130	1,824
Newly infected	98	74	24	41	237
Cured	113	28	164	55	360
Chronically infected	83	59	53	134	329
Total	1,475	397	518	360	2,750

[a]S/P: ratio of sample to positive optical density in the *Staphylococcus aureus*-specific ELISA.
[b]Defined as uninfected = < 0.38 at two sequential herd tests; newly infected = change in S/P ratio from < 0.38 to ≥ 0.38 at sequential herd tests; cured = change in S/P ratio from ≥ 0.38 to < 0.38 at sequential herd tests; or chronically infected = S/P ratio ≥ 0.38 at two sequential herd tests.
[c]Defined as uninfected = <150,000 cells/mL at the preceding and current herd test; newly infected = <150,000 at the previous herd testing and ≥150,000 cells/mL at the current herd test; cured = ≥150,000 at the previous herd test and <150,000 cells/mL at the current herd test; and chronically infected = ≥150,000 at the preceding and current herd test.

Table 4. Results of a linear mixed model of the relationship between milk solids production (kg fat + protein/cow/day) and the S/P[a] ratio category, herd test date, and cow age and breed as fixed explanatory variables for a single, seasonal calving pastoral dairy herd from the Manawatū region of New Zealand, where cows were sampled at herd tests carried out in April 2023, September 2023, December 2023, and April 2024.

				95% CI	
	Coefficient	SE	P-value	Low	High
S/P category[b]					
Neg	Ref.				
Sus	0.013	0.061	0.831	−0.107	0.134
Pos	−0.043	0.050	0.392	−0.141	0.055
Herd test					
Apr 2023	Ref.				
Sep 2023	0.523	0.039	0.000	0.446	0.600
Dec 2023	0.170	0.040	0.000	0.091	0.248
Apr 2024	−0.141	0.043	0.001	−0.226	−0.057
S/P category x herd test					
Neg x Apr 2023	Ref.				
Sus x Sep 2023	0.063	0.065	0.332	−0.064	0.190
Sus x Dec 2023	0.164	0.062	0.008	0.043	0.285
Sus x Apr 2024	0.096	0.064	0.129	−0.028	0.221
Pos x Sep 2023	0.136	0.052	0.009	0.034	0.238
Pos x Dec 2023	0.203	0.051	0.000	0.103	0.304
Pos x Apr 2024	0.125	0.052	0.015	0.024	0.227
Age (years)					
2	Ref.				
3	0.401	0.035	0.000	0.333	0.470
≥ 4	0.549	0.050	0.000	0.450	0.647
S/P category x age (years)					
Neg x 2	Ref.				
Sus x 3	−0.220	0.063	0.000	−0.343	−0.096
Sus x ≥ 4	−0.276	0.053	0.000	−0.381	−0.172
Pos x 3	−0.275	0.054	0.000	−0.380	−0.170
Pos x ≥ 4	−0.420	0.045	0.000	−0.508	−0.332
Breed					
Crossbred	Ref.				
Friesian	0.210	0.042	0.000	0.128	0.292
Intercept	1.109	0.038	0.000	1.035	1.183

[a]S/P: ratio of sample to positive optical density in the *Staphylococcus aureus*-specific ELISA.
[b]Defined as positive (Pos, S/P ≥ 0.38), suspicious positive (Sus, S/P 0.22–0.37), or negative (Neg, S/P < 0.22).
Apr = April; Dec = December; Ref = reference category; Sep = September.

being highest in test-negative cows, intermediate in test-suspicious cows and lowest in test-positive cows. There was also an interaction between S/P status and herd test ($p = 0.006$) whereby there was no difference in MS production by S/P status at the September 2023 and December 2023 herd tests, while at the April 2023 and April 2024 herd tests the test negative cows had higher MS than the test positive cows, with the suspicious group intermediate (Figure 3(b)). Holstein-Friesian cows produced more MS than crossbred cows (1.71 (95% CI = 1.67–1.75) *vs.* 1.52 (95% CI = 1.47–1.57) MS/cow/day for Holstein-Friesians *vs.* crossbreds, respectively; $p < 0.001$), but there was no interaction of breed with S/P category ($p = 0.11$).

In the model of MS production that included S/P status change of cows between two successive herd tests, all the fixed predictors (S/P status change, age and herd test) were significant ($p < 0.001$) along with a significant interaction between S/P status change and age ($p < 0.001$, Table 5). For 2-year-olds, change in infection status had no effect on MS production (Figure 4; Table 6). Among 3-year-old cows, those that cured had higher MS than those cows newly or chronically infected (Figure 4). Among cows ≥ 4 years, newly infected animals produced approximately 0.2 kg/cow/day less MS, while those that cured produced approximately 0.12 kg/cow/day more MS than those cows remaining uninfected. For all age groups, chronically infected cows did not differ in MS from uninfected cows (Figure 4; Table 6).

Discussion

This retrospective longitudinal study is the first to assess associations between *S. aureus* antibody S/P ratios, SCC, and MS production in a pasture-based, spring-calving New Zealand dairy herd. S/P ratio varied by age, stage of lactation and breed. There was a positive association between S/P ratio and SCC, both on a linear and categorical basis. High S/P

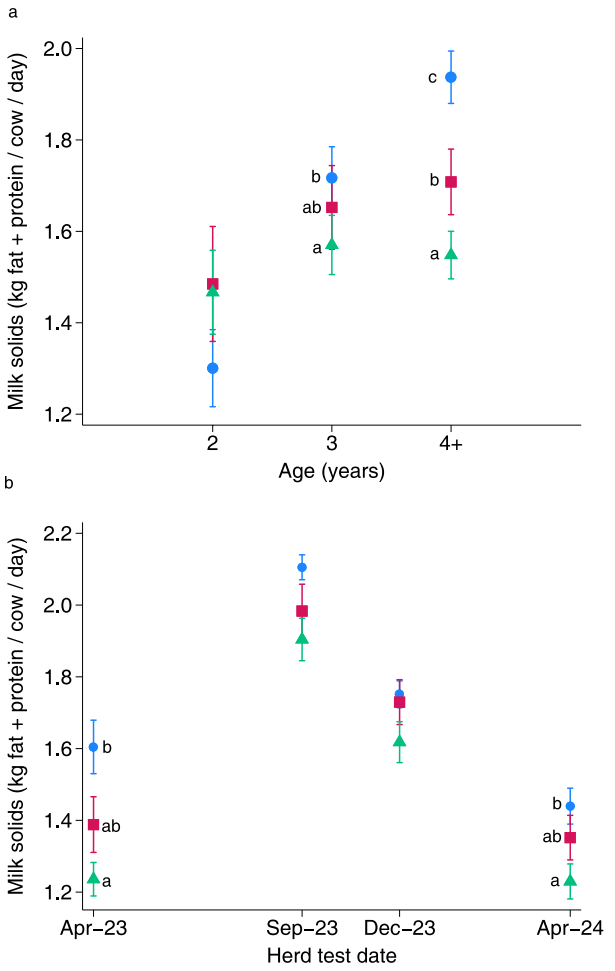

Figure 3. Estimated marginal mean and 95% CI of milk solids production (MS: kg fat + protein/cow/day) across the interaction between the ratio of sample/positive (S/P) optical density in a *Staphylococcus aureus*-specific ELISA (blue circle = test negative (< 0.22), red square = suspicious (0.22–0.37), green triangle = positive (≥ 0.38)) by (a) age for 2-, 3- and ≥ 4-year-old cows; and (b) by herd test date from a single, seasonal calving pastoral dairy herd from the Manawatū region of New Zealand. Within age group and herd test date, points with different superscripts differ at $p < 0.05$.

Table 5. Results of a multivariable model of the relationship between milk solids production (kg fat + protein/cow/day) and the change in S/P[a] category, herd test date and cow age for a single, seasonal calving pastoral dairy herd from the Manawatū region of New Zealand, where cows were sampled at herd tests carried out in April 2023, September 2023, December 2023, and April 2024.

				95% CI	
	Coefficient	SE	P-value	Low	High
Herd test					
Sep 2023	Ref.				
Dec 2023	−0.312	0.018	< 0.001	−0.347	−0.278
April 2024	−0.670	0.019	< 0.001	−0.707	−0.633
Change in S/P category[b]					
Uninfected	Ref.				
Newly infected	0.034	0.051	0.501	−0.066	0.134
Cured	−0.145	0.044	0.001	−0.231	−0.059
Chronically infected	0.108	0.079	0.172	−0.047	0.262
Age (years)					
2	Ref.				
3	0.428	0.042	< 0.001	0.347	0.510
≥ 4	0.702	0.035	< 0.001	0.633	0.772
Change in S/P category x age (years)	Ref.				
Uninfected x 2	Ref.				
Newly infected x 3	−0.194	0.075	0.010	−0.342	−0.046
Newly infected x ≥ 4	−0.236	0.059	< 0.001	−0.353	−0.120
Cured x 3	0.220	0.063	< 0.001	0.097	0.343
Cured x ≥ 4	0.261	0.050	< 0.001	0.164	0.359
Chronically infected x 3	−0.322	0.107	0.003	−0.531	−0.113
Chronic x ≥ 4	−0.229	0.086	0.008	−0.399	−0.060
Intercept	1.539	0.032	< 0.001	1.476	1.601

[a]S/P: ratio of sample to positive optical density in the *Staphylococcus aureus*-specific ELISA.
[b]Defined as uninfected = < 0.38 at two sequential herd tests; newly infected = a change in S/P ratio from < 0.38 to ≥ 0.38 at sequential herd tests; cured = a change in S/P ratio from ≥ 0.38 to < 0.38 at sequential herd tests; or chronically infected = S/P ratio was ≥ 0.38 at two sequential herd tests.

ratios and new infections based on S/P ratio change were associated with reduced milk solids production amongst older cows.

The S/P ratios varied by stage of lactation, with higher values in the first month of lactation, lower through mid-lactation, and increasing thereafter. The high S/P ratio in the first month of lactation may reflect increased antibody levels in general in early lactation milk (Camussone *et al*. 2013), irrespective of infection status. This interpretation is further supported by the observation that for cows in which infection persisted over the dry period, as determined by microbial culture, a specific IgG response in the new lactation was maintained. In contrast, in cows in which the infection was cured, defined by a lack of *S. aureus* isolation, antibodies were only detectable in the first month of the lactation (Fox and Adams 2000). Injection of *S. aureus* capsular antigen conjugated with ovalbumin increased serum Ig for at least 3 weeks (Lee *et al*. 2005) or for several months (Gilbert *et al*. 1994) post-vaccination suggesting some persistence of an IgG response in the absence of active infection. It should be noted, however, that these studies assessed serum, rather than milk IgG, concentrations.

Positive associations were observed between the S/P ratio and SCC, both when considered on a continuous basis (i.e. S/P ratios *vs*. LS; Figure 2) and where the S/P and SCC results were categorised (Tables 2 and 3), which is expected as both are reflective of an inflammatory response (Harmon 1994). However, the agreement between the two tests on a categorical basis was only moderate. This is not unexpected, as the S/P ratio assesses concentrations of specific IgG against *S. aureus*, whereas SCC is a marker of inflammation caused by any IMI (Harmon 1994). Thus, a cow infected with a pathogen other than *S. aureus* would have an elevated SCC in the absence of an increase in the *S. aureus* specific S/P ratio.

The negative impact of intramammary *S. aureus* infections on milk production is well established (Gröhn *et al*. 2004; Gonçalves *et al*. 2018; Heikkilä *et al*. 2018), but the effect of elevated specific antibody levels on production remained to be determined. Our study found that higher *S. aureus* specific IgG levels

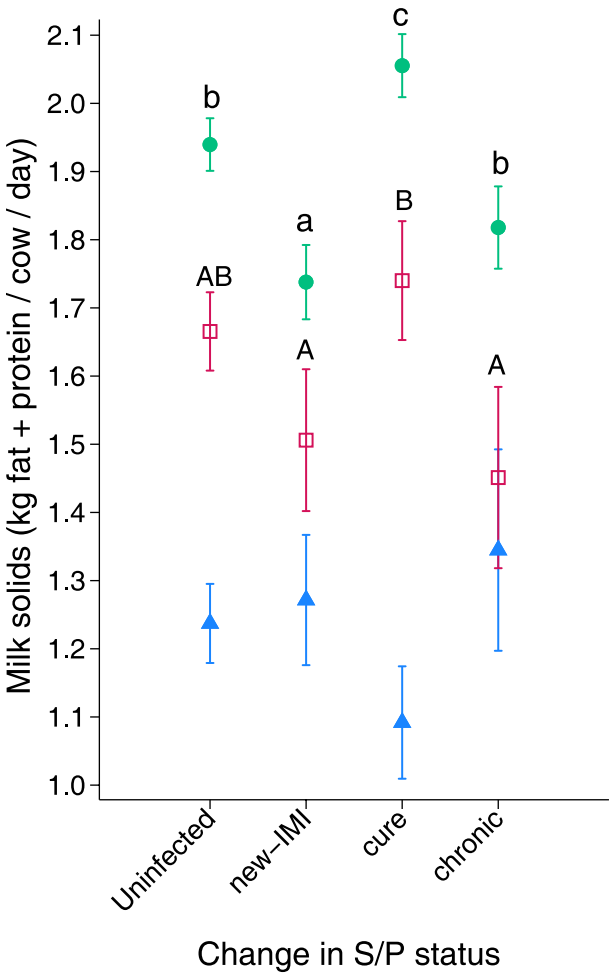

Figure 4. Estimated marginal means and 95% CI of milk solids production (MS: kg fat + protein/cow/day) across the interaction by age (in years on the first of June categorised as 2 (blue triangles), 3 (red squares), or ≥ 4 (green circles) years old), by the change in ratio of sample/positive (S/P) optical density in a *Staphylococcus aureus*-specific ELISA carried out at sequential herd tests carried out between April 2023 and April 2024 in a single, seasonal calving, pastoral dairy herd from the Manawatū region of New Zealand. Change in S/P category between sequential herd tests was defined as uninfected (< 0.38 at two sequential herd tests), newly infected (change in S/P ratio from < 0.38 to ≥ 0.38 at sequential herd tests), cured (change in S/P ratio from ≥ 0.38 to < 0.38 at sequential herd tests), or chronically infected (S/P ratio was ≥ 0.38 at two sequential herd tests). Within age group, values with different superscript differ at $p < 0.05$. The comparisons among 3-year-old animals are indicated by capital letters, while those in ≥ 4-year-old animals are in lower case.

were negatively associated with MS production at a bivariate level (i.e. with no other explanatory variable in the model). When age, herd test, and breed were included in multivariable models, the presence of an interaction between age and S/P category suggested that this relationship held only for cows > 2 years old. S/P-positive cows had lower MS production than test-negative cows in 3- and ≥ 4-year-old cows. Additionally, MS production was depressed in recently infected cows ≥ 4 years old and increased in newly cured cows ≥ 3 years old. S/P-positive cows had significantly lower MS than test-negative cows at the last herd test of each lactation (i.e. April 2023 and April 2024), but there was no difference earlier in lactation (i.e. September 2023 and December 2023). This suggests that the negative impact on MS becomes more evident later in lactation, possibly due to the chronic nature of the infection (Kerro Dego and Vidlund 2024). For reasons that are unclear, there was no association between S/P ratios and MS in 2-year-old cows. This may be related to lower levels of production among 2-year-olds than older animals and hence less impact of infection in this age group; the relatively small sample size of 2-year-olds relative to older cows (they were only about 25% of the study population); and it cannot be excluded that these cows became infected later in the lactation and were higher producing animals at the beginning of the lactation, as high producing cows have a higher incidence of infection (Lescourret *et al.* 1995). Additionally, the effect of mastitis on depression of milk yield is less in first-lactation than later-lactation cows (Gröhn *et al.* 2004).

The external validity of this study is limited as only a single herd was enrolled on a convenience basis. While the associations observed will likely occur in other herds with different mastitis management strategies, bulk milk SCC, and prevalences and incidences of infection, further studies are required to extend the current results. Other limitations of this study include that antibody data was not available for every herd test in the 2023/24 lactation (i.e. only three of the four herd test milk samples were processed via ELISA), and that microbiology data were not available

Table 6. Estimated marginal mean (SE) of milk solids production (kg fat + protein/cow/day) by change in S/P[a] categories and age (years) from a model of the relationship between milk solids production and herd test date, age, and S/P status change between herd tests for a single, seasonal calving pastoral dairy herd from the Manawatū region of New Zealand, where cows were sampled at herd tests carried out in April 2023, September 2023, December 2023, and April 2024.

	S/P status[b]				Difference		
Age (years)	Uninfected	Newly infected	Cured	Chronically infected	Newly infected − Uninfected	Cured − Uninfected	Chronically infected − Uninfected
All	1.74 (0.02)	1.59 (0.02)	1.79 (0.02)	1.65 (0.03)	−0.15	0.05	−0.09
2	1.24 (0.03)	1.27 (0.05)	1.09 (0.04)	1.35 (0.08)	0.03	−0.15	0.11
3	1.67 (0.03)	1.51 (0.05)	1.74 (0.04)	1.45 (0.07)	−0.16	0.08	−0.21
≥ 4	1.94 (0.02)	1.74 (0.03)	2.06 (0.02)	1.82 (0.03)	−0.20	0.12	−0.12

[a]S/P: ratio of sample to positive optical density in the *Staphylococcus aureus*-specific ELISA.
[b]Defined as uninfected = < 0.38 at two sequential herd tests; newly infected = change in S/P ratio from < 0.38 to ≥ 0.38 at sequential herd tests; cured = change in S/P ratio from ≥ 0.38 to < 0.38 at sequential herd tests; or chronically infected = S/P ratio was ≥ 0.38 at two sequential herd tests.

to allow interpretation of the S/P and SCC data in the context of microbiology. It should be noted that microbiology is imperfect, with approximately 90% sensitivity for detection of *S. aureus* in a single milk sample. The StaphGold and SCC tests were performed on cow-composite samples as these are the samples collected at routine herd testing. It is possible that there would be an increase in sensitivity of detection of new infections if quarter- rather than cow-level samples were analysed.

In conclusion, this observational study has demonstrated positive associations between the S/P ratio from the StaphGold test for *S. aureus* and SCC, and negative associations between the antibody levels and MS production amongst older cows and in late lactation. Further validation of these associations using microbiology and investigation of associations between the antibody results and long-term survival in the herd would strengthen the inferences drawn from this study.

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Disclosure statement

A Pernthaner is employed by and a shareholder of Koru Biotech Solutions Ltd (formerly Koru Diagnostics Ltd).

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