Animal, page 1 of 7 © The Animal Consortium 2016
doi:10.1017/S1751731116000227

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Mediation analysis to estimate direct and indirect milk losses associated with bacterial load in bovine subclinical mammary infections

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(Received 14 June 2015; Accepted 8 January 2016)

Milk losses associated with mastitis can be attributed to either effects of pathogens per se *(i.e. direct losses) or to effects of the immune response triggered by the presence of mammary pathogens (i.e. indirect losses). Test-day milk somatic cell counts (SCC) and number of bacterial colony forming units (CFU) found in milk samples are putative measures of the level of immune response and of the bacterial load, respectively. Mediation models, in which one independent variable affects a second variable which, in turn, affects a third one, are conceivable models to estimate direct and indirect losses. Here, we evaluated the feasibility of a mediation model in which test-day SCC and milk were regressed toward bacterial CFU measured at three selected sampling dates, 1 week apart. We applied this method on cows free of clinical signs and with records on up to 3 test-days before and after the date of the first bacteriological samples. Most bacteriological cultures were negative (52.38%), others contained either staphylococci (23.08%), streptococci (9.16%), mixed bacteria (8.79%) or were contaminated (6.59%). Only losses mediated by an increase in SCC were significantly different from null. In cows with three consecutive bacteriological positive results, we estimated a decreased milk yield of 0.28 kg per day for each unit increase in log_2-transformed CFU that elicited one unit increase in log_2-transformed SCC. In cows with one or two bacteriological positive results, indirect milk loss was not significantly different from null although test-day milk decreased by 0.74 kg per day for each unit increase of log_2-transformed SCC. These results highlight the importance of milk losses that are mediated by an increase in SCC during mammary infection and the feasibility of decomposing total milk loss into its direct and indirect components.*

Keywords: tolerance, bovine, mediation analysis, milk loss, mastitis

Implications

During mammary infections, production can be lost directly, indirectly or both. Knowing which losses are the most significant is important for identifying efficient preventive and therapeutic measures and for establishing genetic selection objectives. Here, we propose a mathematical model to estimate these losses using routine information from regional milk recording databases and bacteriological samples. For the specific pathogens found in our study (mostly *Staphylococcus* sp.), indirect milk losses were the most important.

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Introduction

In cattle, subclinical mastitis is a frequent disease, more frequent than the clinical form, that may relapse after a few weeks or last for a long period, and that leads to milk loss (for a meta-analysis, see Seegers *et al.*, 2003). Breeders have two alternatives to decrease such production losses. One is to utilize management practices and/or drugs that reduce the negative effects of mastitis. Another is to select cows more tolerant to the infection, that is, cows able to withstand the infection resulting in minimal loss of milk. Tolerance can be further classified as direct tolerance, that is, the ability to reduce the damages caused by pathogens, and indirect tolerance, that is, the ability to reduce the damages caused by

the immune response triggered by the infection (Schneider and Aires, 2008). The distinction is important for different reasons. One is to identify the most effective treatment protocols. For example, is it necessary to use antibiotics against bacteria or anti-inflammatory drugs to reduce inflammation, or both? Another is to determine selection objectives. These can be different if losses are mainly direct or indirect. If the majority of losses were indirect, then the priority would be to select cattle able to clear an infection without mounting a severe immune response. Conversely, if the majority of losses were not associated with the immune response, then the immune response should be boosted, either pharmaceutically or by selection. If genetic correlations between direct and indirect mechanisms of tolerance are not favorable then improving one type of tolerance mechanisms would worsen the other, thereby negating any benefit. A third reason is that improving direct or indirect processes of tolerance (by genetic selection or preventive practices) may have different effects on the epidemiology of the disease and host–parasite coevolution in the long and short-terms.

By definition, direct and indirect tolerances can be measured by regressing milk yield against an increasing number of pathogens (Kause *et al.*, 2012) and an indicator of the intensity of the immune response to these pathogens (Detilleux *et al.*, 2013), respectively. Higher tolerance is indicated by flatter slopes and lower losses.

Therefore, to estimate both levels of tolerance to mammary infection, we need information on milk yield, level of immune response and bacterial load. Test-day milk yield and somatic cell counts (SCC) are recorded routinely on dairy cows enlisted in national milk recording systems. And, because SCC are constituted mainly of phagocytic cells in infected cows (e.g. Burton and Erskine, 2003), they can be used as an indicator of the intensity of the immune response to mammary pathogens. Bacterial load can be measured by the number of colony forming units (CFU) in culture. However, methods to measure CFU are costly and time consuming, and they are not recorded routinely in field studies.

Mediation models may be appropriate to estimate the effects of mammary infection on test-day milk yields that are mediated or not by test-day SCC. Mediation (VanderWeele, 2012) is the name given to models in which one independent variable *X* (e.g. CFU) affects a second variable *M* (e.g. SCC) which, in turn, affects a third one *Y* (e.g. milk yield). In its linear form, it is also called a three-variable path analysis. The direct effect represents the portion of the relationship between *X* and *Y* that is not transmitted through the intermediate variable *M*, which we will call 'direct tolerance.' The indirect effect represents the portion of the relationship between *X* and *Y* that is transmitted through *M*, which we will call 'indirect tolerance.'

Therefore, the objective of this paper was to perform a mediation analysis to estimate direct and indirect tolerance to bacterial pathogens responsible for subclinical mastitis.

Material and methods

Herds and cows

Three dairy herds were chosen from the coalition group 'Observatory for Udder Health' (OSaM) that federates researchers, dairy associations and breeders to collect information on farm, animal and clinical events in Wallonia (Reding *et al.*, 2011). Herds had accurate and complete information. Cows free from clinical mastitis were sampled at random. Herd, month in milk (stage of lactation), parity and test-day data on udder-composite SCC (*n* cells/10^3 per ml) and milk yield (kg) were extracted from the regional milk-recording database.

Bacteriological samples

From February to April 2013, two surveyors sampled 95 clinically healthy cows, immediately before evening milking. Milk samples were taken three times, 1 week apart, on each cow. The surveyors cleaned teat ends with alcohol swabs and allowed them to dry. They discarded the first few streams and collected milk samples in sterile plastic tubes. Samples were immediately cooled, transported in cool bags to the Bacteriology laboratory of the Veterinary Faculty in Liège, and stored overnight at 4°C.

The procedure for the bacteriological analysis of the milk samples has already been described (Detilleux *et al.*, 2013). Briefly, 1 ml of milk with no macroscopic alteration from each quarter of a cow were pooled and 100 µl of the pool were inoculated onto Columbia base agar (Merck-VWR, Leuven, Belgium) plates supplemented with 5% bovine blood and incubated overnight at 37°C. Counts from duplicate plates were averaged and CFU/ml were recorded as total bacterial load for each pool. Pools with over 100 CFU/ml were marked as 'positive' if a maximum of two types of colonies were detected. Pools with over 100 CFU/ml and more than two colony types were also marked as 'positive' (but contaminated) if one colony type counted for over 100 CFU/ml. Pools with <100 CFU/ml of one/two or of several different colony types were marked as 'negative' or 'contaminated,' respectively. In addition, colonies from 'positive' pools were identified to the genders according to the procedure already described (Detilleux *et al.*, 2013).

Data

For the statistical analyses, three groups of cows were created. The group is called 'NNN' if pools at the three sampling times were all negative, 'PPP' if pools at the three sampling times were all positive, and 'unP' otherwise. Records on milk and SCC were collected up to 3 test-days before and 3 test-days after the date of the first bacteriological sample, within the same lactation. Lactations needed to have at least 5 test-day records, and only the first 300 days in milk were considered. Parities were grouped as 1, 2, 3 and over. The CFU were summed over the 3 sampling dates. Total number of CFU and test-day SCC were expressed in 1000 cells/ml and log-transformed (base 2) so their distributions were closer to normality. Then, one unit increase of $\log_2$-transformed SCC or CFU corresponds to an increase of 2000 cells/ml.

Statistical analyses

The mediation model (Figure 1) is given by the corresponding equations:

$$Z_{ij} = g_0 + g_{1n}T_{ij} + g_{2n}X_i + R_r + M_m + P_p + e_{ij} \quad (1)$$

and

$$Y_{ij} = h_0 + h_{1n}T_{ij} + h_{2n}X_i + h_{3n}Z_{ij} + R_r + M_m + P_p + b_{ij} \quad (2)$$

where Y_{ij} is the test-day milk yield and Z_{ij} the $\log_2$-transformed SCC, T_{ij} the number of days relative to sampling time ($T_{ij} = -40$ to $+40$), i the index for the cow ($i = 1$ to 95) and j the index for the time when milk and SCC were recorded ($j = 1$ to 6). In both equations, n is the index for the group ($n = 1, 2, 3$), X_i the total number of CFU at sampling, R_r the herd ($r = 1$ to 3), M_m is the month in milk at sampling ($m = 1$ to 10), and P_p the parity ($P = 1, 2, 3$). Parameters g_0 and h_0 are the intercepts. Regression coefficients g_{1n} and g_{2n} represent the effect of the number of days relative to sampling and of $\log_2$-transformed CFU on $\log_2$-transformed SCC, respectively. Regression coefficients h_{1n}, h_{2n} and h_{3n} represent the effect of the number of days relative to sampling, of $\log_2$-transformed CFU and of $\log_2$-transformed SCC on test-day milk yield, respectively. These effects were estimated for each group ($n = 1$ to 3) separately. The effects e_{ij} and b_{ij} are independent random variables with normal distributions. Within cows, the covariance structures across repeated errors follow an auto-regressive pattern of order one. The fit of the models was assessed by computing the concordance correlation coefficients between observed and estimated values (Lin, 1989). All interaction terms were not significant ($P > 0.10$) and not included in the final models. Mediation analysis makes the same assumptions of general linear model, including assumptions of linearity, normality and homogeneity of error variance. Measurement errors and the existence of variables affecting both SCC and milk yield could bias the conclusions of the mediation analysis if these were not included in the models.

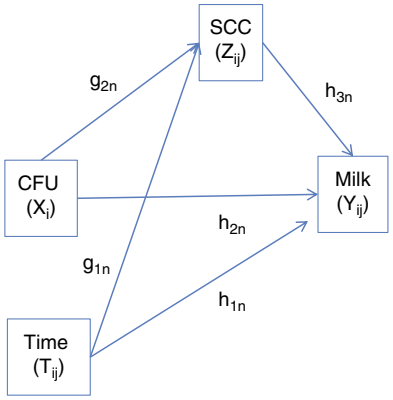

Figure 1 Mediation analysis model. The CFU (X_i) is the $\log_2$-transformed number of bacterial colony forming units, SCC (Z_{ij}) is the $\log_2$-transformed somatic cell counts, time (T_{ij}) is the number of days relative to sampling time, i is the index for the cow, j is the index for the time when milk and SCC were recorded, relative to sampling time. The parameters g_{1n}, g_{2n}, h_{1n}, h_{2n} and h_{3n} are regression coefficients relating independent to dependent variables.

Following the product method (Judd and Kenny, 1981), direct (DE_n) and indirect (IE_n) effects on milk were derived, for each group ($n = 1, 2, 3$) as $DE_n = \hat{h}_{2n}X_i$ and $IE_n = \hat{h}_{3n}\hat{g}_{2n}X_i$. The coefficients $\hat{h}_{2n}$, $\hat{h}_{3n}$ and $\hat{g}_{2n}$ are the REML estimates of the corresponding parameters, adjusted for other effects in the equations. Such effects can be interpreted under the counterfactual framework (Vansteelandt and VanderWeele, 2012): Direct effects measure the change in milk per unit increase in $\log_2$-transformed CFU, as if no change occurrs in $\log_2$-transformed SCC. Indirect effects measure the change in milk yield due to an increase in $\log_2$-transformed CFU that elicits one unit increase in $\log_2$-transformed SCC. Total effects are the sum of the direct and indirect effects, that is, $TE_n = (\hat{h}_{2n} + \hat{h}_{3n}\hat{g}_{2n})X_i$. All effects are adjusted for the effects in model 2. Standard errors were also computed for both direct and indirect effects (Tofighi and MacKinnon, 2011). All analyses were done on SAS Version 9.1 (PROC MIXED) to obtain REML estimates of parameters.

Results

Results of the bacteriological pools are summarized in Table 1. Of the 95 sampled cows, nine were not sampled three times. Out of the 86 remaining cows, 25 were found positive at the three sampling times (PPP) with an average of 5147 (SE = 821) CFU/ml over the three pools; 20 were negative at the three sampling times (NNN) with an average of

Table 1 *Frequency (*n*) and average (SE) of the number of colony forming units (CFU/ml) observed in milk pools from 86 cows*

Group*	Characteristics of the pools	*N*	CFU/ml
NNN	All three pools were bacteriologically negative	20	95 (47)
unP	One pool was bacteriologically positive	22	1246 (2198)
	Two pools were bacteriologically positive	19	1261 (653)
PPP	All three pools were bacteriologically positive	8**	7572 (6148)
		9***	3835 (2386)
		8****	4196 (1871)

*See Material and Methods for information on the groups.
**The three pools were positive for *Staphylococcus* sp.
***Two of the three pools were positive for *Staphylococcus* sp.
****No staphylococci identified.

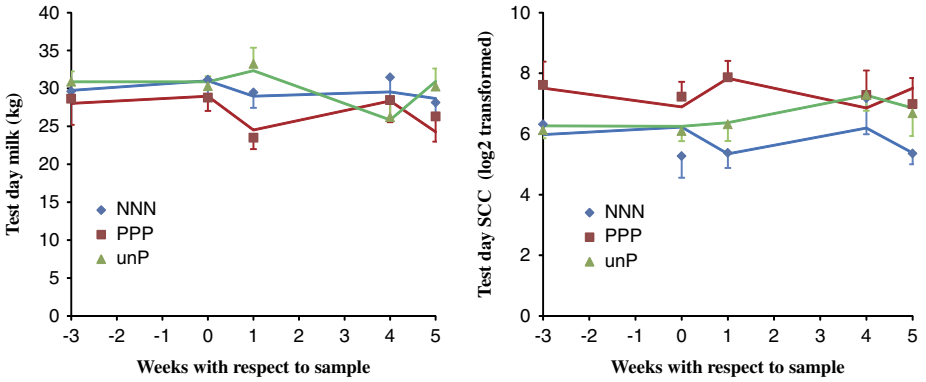

Figure 2 Test-day milk and $\log_2$-transformed SCC for cows with three positive (square), three negative (diamond), and one or two negative (triangle) bacteriological findings. The dots and lines represent weekly means of the observed and estimated data, respectively.

Table 2 *Direct and indirect effects (SE) of $\log_2$-transformed CFU on test-day milk yield for cows with three positive (PPP), three negative (NNN), and one or two negative (unP) bacteriological findings*

	Groups		
Effects	NNN	PPP	unP
Estimates from model 1			
Direct effect of $\log_2$-transformed SCC on test-day milk ($\hat{h}_{3n}$)	0.31 (0.35)	−0.69* (0.28)	−0.74** (0.28)
Direct effect of time on test-day milk ($\hat{h}_{1n}$)	−0.03 (0.03)	−0.01 (0.04)	−0.04 (0.02)
Direct effect of $\log_2$-transformed CFU on test-day milk ($\hat{h}_{2n}$)	0.19 (0.64)	−0.43 (0.72)	−0.61 (0.37)
Estimates from model 2			
Direct effect of time on $\log_2$-transformed SCC ($\hat{g}_{1n}$)	−0.00 (0.01)	−0.01 (0.01)	0.02* (0.00)
Direct effect of $\log_2$-transformed CFU on $\log_2$-transformed SCC ($\hat{g}_{2n}$)	−0.04 (0.11)	0.40** (0.15)	0.12 (0.13)
Estimates from product method			
Indirect effect of $\log_2$-transformed CFU on test-day milk ($\hat{h}_{3n}\,\hat{g}_{2n}$)	−0.01 (0.04)	−0.28* (0.16)	−0.09 (0.11)

CFU = colony forming units; SCC = somatic cell counts.
*$P < 0.05$, **$P < 0.01$.

95 (SE = 47) CFU/ml and 41 were positive at one or two sampling times (unP) with an average of 1253 (SE = 1655) CFU/ml. For SCC, the geometric means were 54 040, 160 875 and 83 970 cells/ml in 'NNN,' 'PPP' and 'unP' cows, respectively. Means (and standard deviations) of milk, $\log_2$-transformed SCC and $\log_2$-transformed CFU were 29.6 (6.90), 6.41 (1.79) and −0.79 (2.30), respectively.

Most bacteriological cultures were negative (52.38%). Others contained either staphylococci (23.08%), streptococci (9.16%), mixed bacteria (8.79%) or were contaminated (6.59%). Considering cows with three positive consecutive samples, we observed staphylococci in at least one sample of 17 cows and in all three samples of eight cows.

A total of 165 test-day records were used for the statistical analyses. Records were from 25 days before up to 39 days after sampling with 1 to 3 records per cow (median = 3). Observed and estimated means of test-day milk and $\log_2$-transformed SCC with respect to the week of sampling are shown in Figure 2 for each group. The fit of the models was better for equations on milk (Lin's concordance coefficient was 88.1%) than for the equations on $\log_2$-transformed SCC (Lin's concordance coefficient was 77.9%). This suggests higher discrepancy between observed $\log_2$-transformed SCC is less.

Estimates of the direct and indirect effects are given in Table 2. In the group 'PPP,' test-day $\log_2$-transformed SCC were higher by +0.40 units for each unit increase in $\log_2$-transformed CFU. This increase prompted a decrease in test-day milk of 0.69 kg for each unit increase in $\log_2$-transformed SCC. The product (0.40×0.69) is 0.28 and corresponds to the (indirect) decrease in test-day milk (in kg) for each unit increase in $\log_2$-transformed CFU that elicited one unit increase in $\log_2$-transformed SCC. The (direct) decrease in test-day milk associated with one unit increase in $\log_2$-transformed CFU but not mediated by an increase in SCC (h_2 in the Table 2) was not significantly different from null. In the group 'unP,' test-day $\log_2$-transformed SCC increased by 0.02 units per day and test-day milk decreased by 0.74 kg for each unit increase of $\log_2$-transformed SCC. However, test-day milk and $\log_2$-transformed SCC were not significantly influenced by the values of $\log_2$-transformed CFU. Thus, direct and indirect milk losses associated with one unit increase in $\log_2$-transformed CFU were not statistically different from null. No significant direct or indirect losses were observed in the group 'NNN.' All other factors in the model (herd, parity and month in milk) significantly affected test day milk.

Total milk losses, that is, the sum of direct and indirect effects, were estimated at 0.71 and 0.69 kg for each unit increase of log_2-transformed CFU for cows in the 'PPP' and 'unP' group, respectively. This implies a loss of 8.7 kg for cows with 5147 CFU/ml, that is, the average of 'PPP' cows (Table 1) and 7.1 kg for cows with 1253 CFU/ml, that is, the average of 'unP' cows (Table 1). These losses are adjusted for the effects in model 2, that is, month in milk at sampling, number of days relative to sampling time, parity and herd.

Discussion

Making the distinction between milk losses due to mammary pathogens or due to the response of the host to them is important for identifying optimal approaches for treating and preventing underperformances associated with intra-mammary infections (Detilleux *et al.,* 2015). On the one hand, bacteria release toxins that may destroy mammary epithelial cells and damage milk-producing tissues. They may also invade and multiply within the bovine mammary epithelial cells before causing cell death (Zhao and Lacasse, 2008). A therapeutic approach against such damages is to milk cows and to give them antibiotic treatment. Cows able to rapidly reduce the number of pathogens present in the gland, for example through a medically boosted or a naturally operational immune response, are also directly tolerant to bacterial injuries. On the other hand, mammary epithelial cells may be injured by products released during the immune response, products against which antibiotics are ineffective. During the immune response, neutrophils migrate from blood capillaries into gland secretions. These are the most abundant and the most important phagocytes of the innate immunity and they constitute more than 90% of somatic cells in cows with clinical mastitis (Sharma *et al*., 2011). If neutrophils are effective as antimicrobial defences, they are also a putative source of molecules with proinflammatory and proteolytic roles which harm the mammary epithelium (Capuco *et al*., 1986; Mehrzad *et al*., 2005). In such cases, a therapeutic approach is to treat cows with anti-inflammatory agents (McDougall *et al*., 2009). Another method would be to breed animals whose neutrophils are best able to kill bacteria (Detilleux *et al*., 1995) so that few somatic cells are necessary to kill pathogens, or animals with superior antibody-mediated immune responses (Thompson-Crispi *et al*., 2012) so they do not need to rely on their cellular immunity, or animals with beneficial anti-oxidant defenses and tissue repair mechanisms (Lauzon *et al.,* 2005).

Here, we evaluated the importance of total, direct and indirect milk losses from field data and estimated the effects of CFU on test-day milk and SCC measured up to 3 weeks before and after bacteriological samples. In both groups of infected cows ('PPP' and 'unP'), direct losses were null while losses mediated by an increase in SCC were significantly greater than null. This observation confirms previous results in cows with notable SCC changes before and after a clinical case (Detilleux *et al*., 2013). Even though SCC remained below the threshold of 200 000 cells/ml considered as normal (Dohoo *et al*., 2001), test-day milk losses were estimated at around 0.7 kg (95%CI: 0.1 to 1.3) per unit increase in log_2-transformed SCC (Table 2). This is within the range of estimates obtained by Hagnestam-Nielsen *et al.* (2009), that is, from 0.2 to 1.2 kg per log_2-transformed SCC in cows free of clinical mastitis. In another study, test-day milk losses varied from 0.34 to 1.35 kg per unit increase in log_2-transformed SCC, according to the pathogen species (Detilleux *et al*., 2015).

Cows in the 'PPP' group were suspected to be chronically infected because they had three consecutive bacteriological positive samples, following the definition of Leitner *et al.* (2000). Compared to the other groups, log_2-transformed SCC in these 'PPP' cows remained at the highest values at all test-days (Figure 2) and effect of time on log_2-transformed SCC was not significantly different from null (Table 2). Such a long lasting high SCC response is typical of *Staphylococcus aureus* infection (Leitner *et al*., 2000), a bacterial species found in 17 out of our 25 'PPP' samples (Table 1). Within the 'PPP' group, cows with the highest CFU load presented the highest SCC and lowest test-day milk. Similarly, Reksen *et al.* (2007) observed a higher decrease in test-day milk yield in cows subclinically infected with >1500 CFU/ml of *S. aureus* than in cows infected with <1500 CFU/ml. Total losses were estimated at 8.7 kg (for the average CFU of 'PPP' cows). Gröhn *et al.* (2003) and Hertl *et al.* (2014) observed a drop of >8 kg in the week following a first case of clinical mastitis due to *S. aureus*.

Cows in the 'unP' group were suspected to be a mix of cows either recently infected or recovering from an earlier infection because only one or two of the three bacteriological samples were positive. In this group, regression coefficients for time on test-day milk and SCC were significantly different from null while the level of CFU was not associated with both test-day values (Table 2). It is well known that SCC increase more or less rapidly at the start of mammary infection (Burvenich *et al.,* 1994). For example, Leitner *et al.* (2000) observed, after intra-cisternal inoculation of *S. aureus* or *Escherichia coli*, a higher SCC increases in cows with acute than chronic infections. Similarly, we observed a higher SCC increase in the group 'unP' than in the group 'PPP.' Total losses in test-day milk yield were estimated at 7.1 kg (for the average CFU in 'unP' cows). Halasa *et al.* (2009) reported a loss of 0.31 kg in primiparous (and 0.58 kg in multiparous) cows for which SCC was >100 000 cells/ml after a test day with SCC <50 000 cells/ml, that is, suspected to suffer from a new subclinical mastitis.

Findings in the present article should be interpreted and used with caution and confirmed in other studies since ours has obvious limitations. A first one is the smallness of the data sample due to financial and personnel restrictions. According to Fritz and MacKinnon (2007), 74 records would have been needed to reach a 80% power for (moderate) effect sizes of the same amplitude as the one observed in this study. Indeed, a completely standardized indirect effect (Preacher and Kelley, 2011) can be computed as the product of the indirect effect size by the ratio of the standard

deviations of CFU on milk. For example, completely standardized indirect effect of total CFU on milk was estimated at 0.09 (i.e. $2.30/6.90 \times 0.28 = 0.09$) in 'PPP' cows, which is considered as 'moderate' by Kenny and Judd (2014). Another limitation is the impossibility to construct pathogen-specific models because infection by the same pathogen was observed in only eight consecutive cultures samples (Table 1). Even if *S. aureus* was present in almost 50% of infected samples, this is unfortunate as pathogen species have different effects on SCC trends (de Haas *et al.*, 2002). Other limitations are linked to the model assumptions which are necessary for obtaining unbiased estimates of the indirect effects. They consist in having uncorrelated error terms, linear relationships, no interaction terms and no unmeasured confounding (Ten Have and Joffe, 2012). Here, effects of CFU on test-day milk and SCC were adjusted for the effects of potential confounders, that is time (T), herd (R), stage of lactation (M) and parity (P). Indeed, it was shown in numerous studies that milk losses associated with increased SCC are most extensive in late lactation and late parities (e.g. Hagnestam-Nielsen *et al.*, 2009). However, we cannot rule out the presence of unmeasured confounders that would have biased one or another relationship. Finally, due to budget constraints, information on test-day CFU (i.e. at the same time SCC and milk were collected) was unavailable and was replaced by information on CFU observed at three consecutive dates. If we had obtained information on test-day CFU, the mediation model would have included two 'causally ordered' mediators (CFU and SCC), with test-day CFU affecting test-day SCC (VanderWeele and Vansteelandt, 2014). With such model, it is possible to compute eight estimates of milk loss not mediated by any changes in SCC and CFU, eight estimates of milk loss mediated only by changes in CFU, eight estimates of milk loss mediated by changes in SCC alone and eight estimates of milk loss mediated by changes in both SCC and CFU (Albert and Nelson, 2011). Suggestions to reduce such complexities have recently been proposed by Daniel *et al.* (2015)

Conclusions

In this study, milk loss due to infection by mastitis pathogens was decomposed into its direct and indirect components in cows tested three times for bacteriological cultures at one week interval. For the specific pathogens found in our study, mostly *Staphylococcus* sp., results stress the importance of milk loss mediated by an increase in SCC in cows free of clinical signs but suspected to be chronically infected. If proven in studies with larger sample sizes than ours, such cows should be treated with anti-inflammatory agents and selection goals for better (indirect) tolerance should be for animals whose neutrophils are best able to kill bacteria, animals with superior antibody-mediated immune responses, and/or animals with beneficial anti-oxidant defenses and tissue repair mechanisms.

Acknowledgment

This study was supported by EADGENE (European Animal Disease Genomics Network of Excellence for Animal Health and Food Safety).

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