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Associations between subclinical intramammary infection and milk fatty acid profile at the quarter level in Holstein cattle

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ABSTRACT

Mastitis, especially the subclinical form, is the most common economic and health problem in dairy cows. Little is known about changes in milk fatty acid (**FA**) composition according to infection/inflammation status of the mammary gland. The aim of this study was to investigate the associations between naturally occurring subclinical intramammary infection (**IMI**) from different pathogens, i.e. *Streptococcus agalactiae*, *Staphylococcus aureus*, *Streptococcus uberis* and *Prototheca* spp., and the detailed milk FA profile assessed at quarter level in Holstein cows. After an initial bacteriological screening (**T0**) on 450 Holstein cows reared in three dairy herds, we identified 78 cows positive at the bacteriological examination. These animals were followed up at the quarter level two weeks (**T1**) and six weeks (**T2**) after T0. In total, 600 single-quarter samples were obtained at T1 and T2. Individual FAs were determined using the gas chromatography analytical method. Investigated traits were 70 individual FAs, 12 FA groups, and six desaturation indices. The associations between subclinical IMI combined with somatic cell count (**SCC**) and milk FA profile were investigated using a hierarchical linear mixed model (i.e., observational unit was quarter within cow) with the following fixed effects: days in milk (**DIM**), parity, herd, SCC, bacteriological status (**BACT**, positive and negative), and the SCC × BACT interaction. The random effect of individual cow nested within herd, DIM and parity was used as the error term for the latter effects. The most significant associations were detected at T2. Notably, IMI reduced the proportions of individual short-chain FA, especially 4:0 and 6:0 (−14%), but increased the proportion of the most abundant medium-chain FA (**MCFA**), 16:0 (+4%). A reduction in the desaturation indices was observed mostly for 14:1 index (−9%), in line with the reduction in 14:1 (−10%). Somatic cell count significantly affected 14 individual FAs. In particular, samples with high SCC (≥200 000) had significantly lower proportions of 8:0, 10:0, 11:0, 12:0, and 13:0 compared with samples with low SCC (<200 000). Increasing SCC in animals positive at the bacteriological examination were associated with a reduction in total MCFA at T2 (while in negative animals, they remained constant across SCC classes), possible evidence that elongation of the FA chain from 11 to 16 carbons is affected by a combination of infection and SCC. This study showed that subclinical IMI and SCC are mainly associated with reductions in the synthesis of FA and the desaturation process in the mammary gland.

Implications

We assessed the associations between naturally occurring subclinical intramammary infection combined with somatic cell count and the milk fatty acid profile in Holstein cows. Our results showed that the profiles of fatty acids originated by *de novo* synthesis and desaturation processes were the most affected. From a practical point of view, this information could help for the development of predictive tools. In terms of scientific impact, it could pinpoint potential pathways involved in the metabolic response of the mammary gland to the infection/inflammation challenge.

Introduction

Mastitis is still the most frequent and challenging disease in dairy cows, particularly in its subclinical form, which is difficult

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to detect since it does not cause any visible changes in milk or udder appearance. Bacteriological examination is still the gold standard method for identifying infected animals and the pathogen causing the intramammary infection (**IMI**). Somatic cell count (**SCC**) is however routinely used to detect potential cases of subclinical mastitis and to define milk quality standards at the farm level. A threshold of 200 000 cells/mL in quarter or composite milk samples is currently widely accepted as an indicator of subclinical disease (NMC, 2017). With regard to SCC, it does not always reflect the actual health condition of the animal (i.e. infected animals can have low SCC, and culture-negative animals have high SCC), especially when taking composite milk and not mammary quarters into account (Hogan et al., 1989; Berglund et al., 2004).

Several studies have been published on the detrimental effects of clinical or subclinical IMI on milk components (Hogarth et al., 2004; Forsbäck et al., 2009; Bobbo et al., 2017; Stocco et al., 2019; Addis et al., 2020), and on milk coagulation properties and cheese-making traits (Bobbo et al., 2016; Pegolo et al., 2021). The relationships between IMI and milk fat, however, have received little attention. A reduction in the fat percentage has been reported, while free fatty acids (**FAs**) seem to increase in mastitic milk due to the presence of leucocyte lipases (Le Maréchal et al., 2011). Several studies investigated the complex molecular mechanisms underlying milk fat synthesis and metabolism within the bovine mammary gland (Bionaz and Loor, 2008) and their changes associated with subclinical IMI (Moyes et al., 2009; Xu et al., 2016; Weller et al. 2019).

As regards variations in milk FA composition induced by IMI, studies are limited to a few single FA determined on composite milk (Ali et al., 2022). However, Paixão et al. (2017) reported that the single-quarter case of mastitis can influence milk composition in adjacent quarters, which underlines the need to investigate the relationship between IMI and milk fatty acid composition at the level of the quarter. In this context, Turini et al. (2020) evaluated the relationships between SCC as an indicator of inflammatory conditions and the milk FA profile in single udder quarters, but they do not provide any information on the animals' bacteriological status.

To fill this gap, the aim of this study was to evaluate the associations between naturally occurring subclinical IMI infection caused by different pathogens, namely *Streptococcus agalactiae*, *Staphylococcus aureus*, *Streptococcus uberis* and *Prototheca* spp., combined with SCC and 70 individual FAs, 12 FA groups and six desaturation indices analysed by gas chromatography at the quarter level in Holstein cows.

Material and methods

Study design, animals and sampling

This research was approved by the Ethical Committee for the Welfare and Use of Animals (OPBA – Organismo Preposto al Benessere degli Animali) of the Università Cattolica del Sacro Cuore, and by the Italian Ministry of Health (protocol number 510/2019-PR of 19/07/2019). This research was a longitudinal observational study aimed at determining the associations between milk FA, determined at the quarter level, and subclinical IMI combined with SCC. The null hypothesis was that no association existed between them. The observational unit was the mammary quarter within cow.

Sample size was calculated assuming that short-chain FA (**SCFA**) could be the ones most influenced by udder health status, especially those of the *de novo* synthesis within mammary gland (Turini et al., 2020). This hypothesis is supported by the fact that infection can damage the mammary epithelial cells and therefore affect milk FA synthesis. Therefore, the sample size was calculated to achieve a 95% confidence and 80% power to detect differences of at least 1.2 g/100 g of SCFA, corresponding to roughly 30% of the SD of the trait between culture-negative and culture-positive milk samples. Considering an expected prevalence of animals affected by subclinical IMI of around 20% (Zecconi et al., 2019) and the variability of milk FA profile, we considered 280 quarters enough to detect the desired differences (SAS Institute Inc., Cary, North Carolina, USA).

Milk samples were collected from 450 lactating Holstein cows belonging to three different commercial farms in the Veneto region (northern Italy; 144, 71, and 235 in herds A, B, and C, respectively). Management and farming conditions were previously reported in detail in Pegolo et al. (2021). Briefly, herds were selected on the basis of the results of a previous survey carried out by the Istituto Zooprofilattico delle Venezie (IZSVe) on the herd-prevalence of *Streptococcus agalactiae*, *Staphylococcus aureus*, *Streptococcus uberis* and *Prototheca* spp. in the Veneto region. Herds characterised by the highest prevalence of these pathogens were selected. Cows were housed in free stalls and fed TMR based mainly on corn silage, sorghum silage, and concentrates, and milk was collected twice a day. Feed ingredients and main nutritional values of the rations used are reported in Supplementary Table S1. Drinking water was available in automatic water bowls.

The longitudinal observational study was carried out in three sampling periods, which were selected considering the intermittent shedding pattern of some of the pathogens, e.g. *Staphylococcus aureus* (Sears et al., 1990). Composite milk samples (50 mL) were aseptically taken from all lactating cows in each herd (T0, n = 450), and subjected to bacteriological screening to identify healthy animals and those with IMI. Following this screening, we selected 78 animals positive at the bacteriological examination with no clinical signs of mastitis and no history of treatment with antibiotics. Subsequent to the initial sampling (**T0**) for bacteriological screening, two 50 mL aliquots of milk were taken from each quarter of the selected positive animals two (**T1**) and six weeks (**T2**) after T0 to monitor the course of the disease (n = 600) (Supplementary Fig. S1). One aliquot was taken to the Milk Quality Laboratory of The Breeders Association of the Veneto Region (ARAV) for determination of SCC and analysis of milk composition. The other aliquot was taken to the Laboratory of the Department of Agronomy, Food, Natural Resources, Animals and Environment (DAFNAE) of the University of Padova for analysis of the milk FA profile by gas chromatography. All milk sampling was performed in accordance with National Mastitis Council guidelines (NMC, 2017).

Microbiological analysis

Microbiological analysis of composite and quarter milk samples was carried out at the IZSVe. Samples were frozen after delivery to the laboratory and analysed within three days. Ten µL of milk from the composite samples was inoculated on each of the following selective media: Baird-Parker agar with rabbit plasma fibrinogen (BP-RPF**,** Biokar Diagnostic, Beauvais, France), thallium sulphate-crystal violet-B toxin blood agar (**TKT,** IZSVe internal production), and *Prototheca* isolation medium (**PIM**, IZSVe internal production). Baird-Parker agar with rabbit plasma fibrinogen plates were prepared according to ISO 6888-2 (1999), TKT according to the method described by Hauge and Ellingsen (1953), and PIM to National Mastitis Council guidelines (NMC, 2017). Inoculated media were incubated at 37 °C. Baird-Parker agar base plates were observed for growth at 24 and 48 h of incubation. Suspected *Staphylococcus aureus* colonies were confirmed by tube coagulase test (NMC, 2017). Thallium sulphate-crystal violet-B toxin blood agar plates were observed at 24 h, and suspected colonies of *Streptococcus agalactiae* were confirmed by CAMP test (NMC, 2017).

Prototheca isolation medium plates were observed at 24, 48 h, and 72 h, and suspected colonies were confirmed by the wet mount method (NMC, 2017).

Quarter milk samples were cultured according to NMC guidelines (NMC, 2017). In brief, 10 µl of milk was streaked on blood esculin agar, and after incubation at 37 °C for 16–18 h single isolated colonies were picked, subcultured and identified by morphological examination and a supplementary test according to NMC guidelines (2017). For mixed species cultures, the prevalent cultures were isolated and examined up to a maximum of five different colonies (in case of more than five colonies, the sample was labelled as contaminated). Culture-positive status was attributed in the presence of at least one colony-forming unit/plate (CFU/-plate). The same criteria were used for all the microorganisms both at composite (T0) and quarter level (T1 and T2). Bacterial colonies with doubtful or inconsistent test results were identified by matrix-assisted laser desorption ionisation–time of flight mass spectrometry performed by Microflex Biotyper LT (Bruker Daltonics GmbH, Bremen, Germany). For calibration, the Bacterial test Standard was used according to the standard manufacturer's instructions. The threshold for species-level identification was a log (score) ≥2.0.

Milk fat and quality traits, and milk fatty acid analysis

Milk fat and quality traits, and milk FA analyses were carried out on quarter milk samples. Milk fat percentages were determined with an FT6000 Milkoscan infrared analyzer (Foss A/S, Hillerød, Denmark). Somatic cell counts (cells/mL) were provided by a Fossomatic™ 7 DC analyzer (Foss A/S, Hillerød, Denmark). The analytical procedure for the determination of the FA profile on quarter milk samples is described in detail in Giannuzzi et al. (2022). Briefly, a variable amount was taken from each freeze-dried milk sample to obtain 40 mg of lipid. Before gas chromatography injection, the samples were converted to methyl esters through direct transesterification of all classes of milk lipids using a variety of acid- and base-catalysed processes, according to Christie (1993). The procedure consisted in two successive steps: First, each sample was heated in a thermal bath to 50 °C with 1 mL of sodium methoxide (0.5 M in methanol), then cooled; second, 1.5 mL of 5% methanolic HCl was added and the samples were heated to 80 °C, then cooled. Finally, 2 mL of hexane and 2.5 mL of 6% K_2CO_3 were added to each sample. The resulting solution was centrifuged and the supernatant collected for gas chromatography. Separation of milk FA was performed with a 7820A GC System (Agilent Technologies, CA, USA) fitted with an automatic sampler (Agilent 7693, CA, USA), SLB-IL 111 columns (100 m × 0.25 mm, 0.2 µm film thickness; Merck KGaA, Darmstadt, Germany) and a flame ionisation detector (FID) connected to chromatography data system software (OpenLab CDS, Agilent ChemStation, CA, USA). Timings, temperature and flow rate were as used by Giannuzzi et al. (2022). The internal standard technique was used to quantify the fatty acid methyl esters. The area of each FA was corrected using flame ionisation detector relative response factors (Schiavon et al., 2016). A calibration procedure based on five serial dilutions for each of the 38 standard FA purchased (1, 0.5, 0.25, 0.125, and 0.0625) was performed to compute the response factor values. The resulting 38 calibration curves were all linear with R^2 values >0.997. Each FA was quantified as an adjusted area with respect to the total adjusted area of all FAs (excluding the area of the internal standard, methyl 12-tridecenoate) in the chromatogram, and was expressed in g/100 g of total FA, based on the concentration in weight of the methyl 12-tridecenoate in the solution.

To establish empirically if a peak was measurable, we took the 38 dilution curves having residual SDs ranging from 0.0007 to 0.0180 mg, with a mean of 0.005 mg. In accordance with previous studies (Pegolo et al., 2016), we assumed a single limit of detection for all FAs which was equal to 0.005 mg × 3. Analytical values <0.015 mg (0.0015 g/g total FA) were assumed undetectable and filtered out. In total, 89 fatty acid peaks were identified either by the comparison with 38 commercial standards (Supelco 37 Component FAME Mix (Sigma-Aldrich), (Nu-Chek Prep, Inc. Elysian, MN, U.S.A.), Supelco Conjugated (9Z,11E) – Linoleic acid (Sigma-Aldrich)) and by the comparison with the elution times reported by Delmonte et al. (2012).

Statistical analyses

We carried out a preliminary exploratory analysis of the data to check the assumptions of normality required for model fitting and hypothesis testing (i.e. presence of extreme values, and possible non-normal distribution of the variables). To consider only FA which can have detectable variations, only 53 accounting for >0.05 mg/100 g of the total FA were subsequently analysed. The associations between milk FA at the quarter level and IMI combined with SCC were explored with the SAS PROC MIXED procedure (SAS Institute Inc., Cary, North Carolina, USA). The two sampling time points (T1 and T2) were analysed independently due to the heterogeneity of the variances and to the possible change in bacteriological status of the quarter between T1 and T2. In the following model, the observational unit is the individual mammary quarter:

$$y_{ijklmn} = \mu + \mathrm{DIM}_i + \mathrm{Parity}_j + \mathrm{Herd}_k + \mathrm{SCC}_l + \mathrm{BACT}_m + (\mathrm{SCC} \times \mathrm{BACT})_{lm} + Cow_{n:ijk} + e_{ijklmn} \quad [M1]$$

where y_{ijklmn} is the investigated trait (individual FA, FA group or desaturation index); µ is the overall mean; **DIM**$_i$ is the fixed effect of the ith class of days in milk (i = 3 classes; class 1 ≤ 120; 120 < class 2 ≤ 240; class 3 > 240); Parity$_j$ is the fixed effect of the jth parity (j = primiparous or multiparous); Herd$_k$ is the fixed effect of the kth herd (k = 3 herds); SCC$_l$ is the fixed effect of the lth class of SCC (l = 4 classes; class 1 < 50 000 cell/mL; 50 000 cell/mL ≤ class 2 < 200 000 cell/mL; 200 000 cell/mL ≤ class 3 < 400 000 cell/mL; class 4 ≥ 400 000 cell/mL); **BACT**$_m$ is the fixed effect of the mth class of bacteriological status (m = positive or negative); $(\mathrm{SCC} \times \mathrm{BACT})_{lm}$ is the two-way interaction between SCC$_l$ and BACT$_m$; Cow$_{n:ijk}$ is the random effect of the nth animal nested within DIM$_i$, parity$_j$, and herd$_k$; and e_{ijklmn} is the random residual. Cow and the residuals were assumed to be normally distributed with a mean of zero and variances of σ_r^2 and σ_e^2, respectively. The ratio $\sigma_r^2/\sigma_r^2+\sigma_e^2$ was calculated as a measure of quarter independence (repeatability, %). With this nested design, the effects of DIM$_i$, parity$_j$, and herd$_k$ were tested on the error line of the nth animal nested within DIM$_i$, parity$_j$, and herd$_k$, while the SCC$_l$, BACT$_m$, $(\mathrm{SCC} \times \mathrm{BACT})_{lm}$, and animal effects were tested on the residual term. The effects of the number and position of affected quarters were also tested, but were never significant and were therefore excluded from the final analyses.

Orthogonal contrasts for SCC classes were set as follows:

- class 1 + class 2 *vs* class 3 + class 4 (low SCC *vs* high SCC)
- class 1 *vs* class 2 (within low SCC)
- class 3 *vs* class 4 (within high SCC)

Orthogonal contrasts for the BACT × SCC interaction were set as follows:

- negative (SCC class 1 + class 2 + class 3 + class 4) *vs* positive (SCC class 1 + class 2 + class 3 + class 4)
- negative (low SCC) *vs* positive (low SCC)
- negative (high SCC) *vs* positive (high SCC)

Table 1
Results from linear mixed model (*F*-values and significance) and repeatability[1] at sampling time 1 (T1) and 2 (T2) for the associations between bacteriological status, somatic cell count and their interaction, and milk individual fatty acids at quarter level in Holstein cows.

Item[2]	T1				T2			
	BACT[3]	SCC[4]	SCC × BACT[5]	Rep, %	BACT[3]	SCC[4]	SCC × BACT[5]	Rep, %
Individual FA								
SFA								
4:0	0.08	1.64	2.20	54	11.99***	0.45	0.95	32
6:0	2.30	1.95	2.29	63	13.33***	0.93	0.55	33
8:0	1.82	3.37*	2.15	63	10.57**	2.92*	0.11	32
9:0	5.64*	1.17	0.75	61	4.60*	1.89	0.36	58
10:0	1.63	3.18*	1.87	57	3.40	4.59**	0.76	42
11:0	4.55*	0.88	1.43	64	0.47	3.62*	1.10	64
12:0	2.06	2.60	1.72	55	0.10	4.72**	2.50	56
13:0	0.01	0.34	0.36	65	2.98	3.26*	0.86	69
14:0 iso	0.98	3.91**	0.87	61	0.08	1.68	1.25	57
14:0	0.17	1.02	0.68	60	1.27	4.64**	3.15*	65
15:0 iso	4.91*	3.01*	1.28	60	1.12	2.77*	1.34	60
15:0 ante	12.51***	2.07	1.00	54	0.17	1.2	0.29	60
15:0	0.09	1.20	0.61	68	0.88	0.94	0.88	78
16:0 iso	5.75*	2.82*	1.78	58	10.43**	3.08*	2.07	48
16:0	0.37	0.51	0.38	59	11.62***	0.97	2.19	63
17:0 iso	6.06*	1.10	0.82	54	1.29	1.43	0.18	51
17:0 ante	5.64*	0.34	0.58	63	0.02	3.74*	0.31	51
18.0 iso	0.69	1.79	3.91**	73	7.6**	2.35	1.21	63
18:0	0.46	0.70	0.82	57	2.71	2.2	0.56	71
20:0	0.09	2.25	0.17	51	1.96	1.1	0.92	49
21:0	1.84	0.87	0.79	63	1.17	1.77	0.57	34
23:0	0.65	2.77*	2.13	29	0.38	0.43	0.31	19
MUFA								
10:1 (c9)	2.65	0.94	0.85	61	6.85**	1.02	0.27	43
12:1 (c9)	6.30*	0.36	0.54	71	17.23***	1.74	1.29	54
14:1 (c9)	1.80	0.81	0.04	63	8.03**	1.71	1.40	68
16:1 (t9)	5.64*	0.34	0.58	63	0.02	3.74*	0.31	51
16:1 (c11)	7.70**	0.16	0.71	49	3.59	0.35	0.03	51
18:1 (t6 + t8)	0.84	0.64	0.95	51	0.03	2.09	1.10	29
18:1 (t9)	0.25	2.88*	0.20	63	0.04	2.20	0.98	46
18:1 (t10)	2.67	0.34	0.18	43	7.70**	0.58	0.75	52
18:1 (t11)	0.14	2.92*	0.36	52	4.03*	0.72	1.72	44
18:1 (t12)	0.13	0.22	0.61	48	2.23	0.62	1.04	58
18:1 (c9)	1.81	0.03	0.42	58	0.02	1.17	0.58	53
18:1 (c11)	1.08	0.59	0.20	53	4.35*	1.03	0.47	36
18:1 (c12)	3.16	1.20	0.28	50	5.30*	1.54	0.46	53
18:1 (c13)	4.76*	0.93	1.66	61	9.21**	0.36	0.12	30
18:1 (t16 + c14)	0.05	1.90	0.72	62	1.11	1.54	0.19	50
18:1 (c15)	0.26	0.02	0.25	64	5.45*	0.75	0.83	48
19:1 (t7)	16.15***	0.59	0.19	54	1.62	0.82	0.55	43
20:1 (c9)	4.01*	1.63	1.85	71	1.64	0.25	0.17	35
20:1 (c11)	8.95**	0.51	0.62	66	14.33***	2.18	0.62	58
PUFA								
18:2 (t9, t12)	2.15	1.46	0.19	55	1.58	1.20	1.48	48
18:2 (c9, c12)	0.52	1.63	1.72	60	0.15	0.71	1.26	48
18:2 (t7, c9)	0.46	0.17	0.04	39	7.99**	3.30*	1.29	25
18:2 (c9, t11)	2.61	0.71	0.41	63	0.58	3.38*	2.87*	66
18:3 (c9, c12, c15)	1.45	0.09	4.59**	70	2.12	1.71	0.97	52
20:3 (c8, c11, c14)	0.01	1.48	1.70	40	0.32	1.36	0.41	49
20:4 (c5, c8, c11, c14)	1.40	0.98	1.98	25	0.11	3.75*	2.03	44
20:5 (c5, c8, c11, c14, c17)	0.06	1.19	1.12	37	0.57	0.15	0.09	14
22:5 (c7, c10, c13, c16, c19)	0.29	1.24	2.66*	36	3.88	4.91**	1.11	51

[1] Repetability (%) calculated as ratio between $\left(\frac{\sigma^2_r}{\sigma^2_r+\sigma^2_e}\right) \times 100$, where σ^2_r and σ^2_e, are variances attributed to the effect of replicate-animal and error, respectively.
[2] FAs = fatty acids; SFAs = saturated fatty acids; MUFAs = monounsaturated fatty acids; PUFAs = polyunsaturated fatty acids; c = cis; t = trans.
[3] BACT = Bacteriological status, expressed in classes (positive; negative).
[4] SCC = somatic cell count, expressed in classes (four classes; class 1 < 50 000 cell/mL; 50 000 cell/mL ≤ class 2 < 200 000 cell/mL; 200 000 cell/mL ≤ class 3 < 400 000 cell/mL; class 4 ≥ 400 000 cell/mL).
[5] BACT × SCC = interaction between the two main effects.
* $P < 0.05$.
** $P < 0.01$.

- negative *vs* positive (SCC class 1)
- negative *vs* positive (SCC class 2)
- negative *vs* positive (SCC class 3)
- negative *vs* positive (SCC class 4)

The significance threshold was set at $P < 0.05$.

Results

Microbiological analyses and milk fatty acids

A detailed description of the results of the microbiological analyses at the cow level, and herd-prevalence are reported in Pegolo

et al. (2022). Briefly, at the initial bacteriological screening, the following microorganisms were isolated: *Staphylococcus aureus* (n = 6 animals from 2 herds), *Streptococcus agalactiae* (n = 51 animals from 2 herds), *Prototheca* spp. (n = 19 animals from 1 herd) and *Streptococcus uberis* (n = 5 from 1 herd). There were no differences in milk production and milk composition between cows that were negative and cows that were positive at bacteriological examination, except for SCC. Somatic cell count average values were higher in the latter group (199 700 vs 688 600 cells/mL).

Milk FA profiles were determined for a total of 600 quarter milk samples: 314 at T1 and 286 at T2. Twelve samples were contaminated from environmental microorganisms at T1 and 10 at T2, and were therefore excluded from the statistical analysis. The most prevalent pathogen was *Streptococcus agalactiae* at both T1 (n = 52, 17 %) and T2 (n = 36, 13%) (Supplementary Table S2). At T1, three of the quarter samples were positive for both *Streptococcus agalactiae* and *Prototheca* spp. At T2, two of the quarter samples were positive for both *Streptococcus agalactiae* and *Prototheca* spp., and one for both *Streptococcus agalactiae* and *Staphylococcus aureus*. In total, we found 86 quarters positive at the bacteriological examination at T1, and 57 at T2. From T1 to T2, 36 quarters changed the infection status from culture-negative to culture-positive and 49 from culture-positive to culture-negative. Sixteen quarter samples collected at T1 were not present at T2 due to cows culling. At T1, 23 animals had one culture-positive quarter and 62 had more than one culture-positive quarters. At T2, 23 animals had one culture-positive quarter and 34 had more than one culture-positive quarters. Milk FA composition is reported in Supplementary Tables S3 and S4. Overall, the FA proportions assessed on quarter samples with the same methodology were in line with previous data on composite samples (Mele et al., 2016).

Associations between subclinical intramammary infection and milk fatty acids

At T1, bacteriological status was associated with 14 individual FAs, n3-FA, and the n6/n3 ratio ($P < 0.05$) (Table 1; Fig. 1; Supplementary Figs. S2–S4). In particular, subclinical IMI was associated with higher proportions of 9:0 (+11%) and 11:0 (+9%) (Supplementary Fig. S2) and lower proportions of several branched-chain FA (**BCFA**), especially 16:0 iso (−8%) (Supplementary Fig. S3). Among the monounsaturated FA (**MUFA**), milk from IMI quarters had higher proportions of 16:1 c11 (+23%) and 18:1 c13 (+15%) (Supplementary Fig. S4). The n3-FA category was lower in milk fat from IMI quarters (−3%) with a consequent increase in the n6/n3 ratio (+3%) (Fig. 1).

At T2, subclinical IMI was associated with the profile of 18 individual FAs, SCFA, medium-chain FA (**MCFA**), and the 10:1, 14:1 and 18:1 indices ($P < 0.05$) (Tables 1 and 2; Fig. 1; Supplementary Figs. S2–S4). In particular, milk fat from IMI quarters had lower proportions of several individual SCFAs, especially 4:0 and 6:0 (−14%), and the proportion of the most abundant MCFA, 16:0, was higher (+4%). Among the individual BCFA, 16:0 iso and 18:0 iso had opposite patterns: the former was higher in milk fat from IMI quarters (+17%), the latter was lower (−11%) (Supplementary Fig. S3). Intramammary infection was also associated with reductions in several individual MUFA, especially 20:1 c11 (−46%) and 18:1 c13 (−21%) (Supplementary Fig. S4). Among the individual PUFA, only the proportion of CLA 18:2 t7, c9 was higher (+14%). Overall, in milk fat from IMI quarters, the SCFA category was 11% lower, while the MCFA category was 3% higher (Fig. 1). A reduction in the unsaturation indices was observed in particular for the 14:1 index (−9%) (Fig. 1) in culture-positive samples respect to culture-negative samples, which was in line with the reduction in the corresponding product (14:1, −10%). Overall, repeatability was generally moderate (between 30 and 60%), except for those FAs which

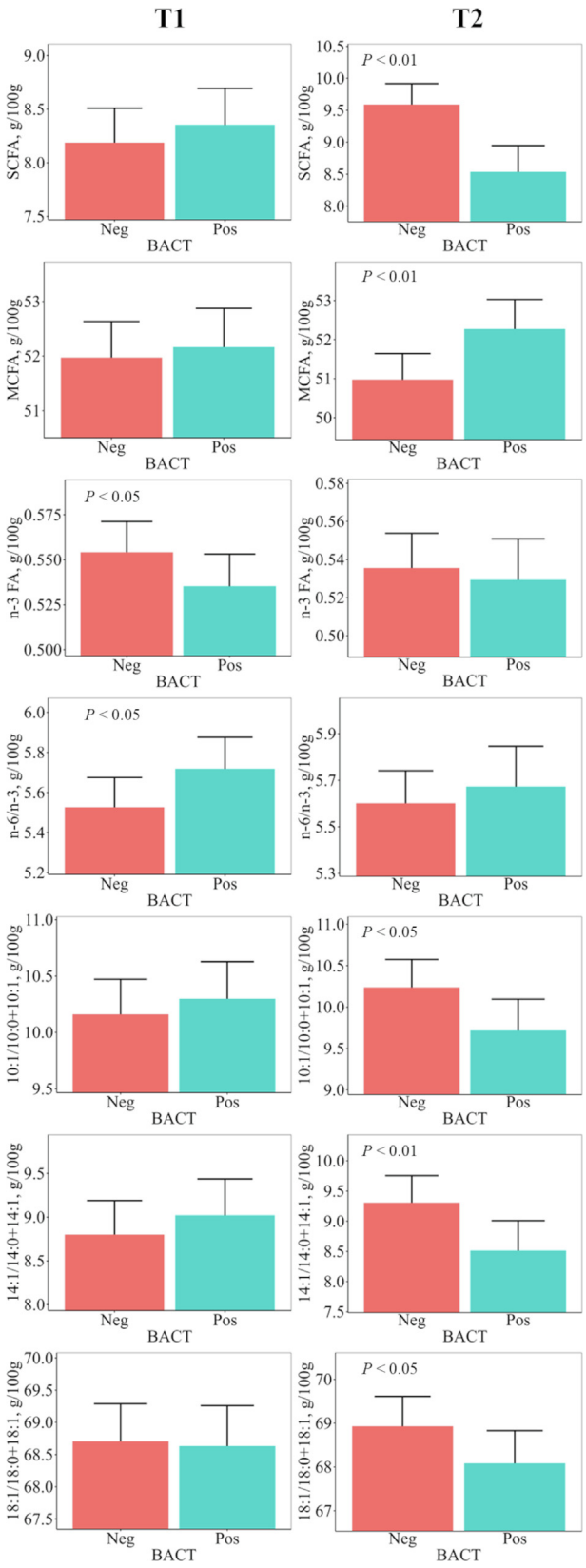

Fig. 1. Least square means and SE of fatty acid groups and unsaturation indices according to bacteriological (BACT) status classes in Holstein cows. BACT classes were defined as: negative at bacteriological examination (neg) and positive at bacteriological examination (pos). SCFAs: short-chain fatty acids; MCFAs: medium-chain fatty acids; n3 FAs: omega-3 fatty acids; T1: sampling time 1 (2 weeks after T0); T2: sampling time 2 (4 weeks after T1). Only significant ($P < 0.05$) traits for at least one sampling time were displayed.

Table 2
Results from linear mixed model (*F*-values and significance) and repeatability[1] at sampling time 1 (T1) and 2 (T2) for the associations between bacteriological status, somatic cell count and their interaction, and milk group of fatty acids at quarter level in Holstein cows.

Item[2]	T1				T2			
	BACT[3]	SCC[4]	SCC × BACT[5]	Rep, %	BACT[3]	SCC[4]	SCC × BACT[5]	Rep, %
Group of FA								
SFA	1.64	0.16	0.64	59	1.12	1.30	0.63	54
MUFA	1.36	0.12	0.50	58	1.18	1.13	0.42	56
PUFA	0.22	2.86*	2.26	60	0.37	1.20	0.98	48
SCFA	0.77	3.10*	2.81*	61	11.07**	2.12	0.09	33
MCFA	0.21	0.32	0.13	56	8.38**	2.56	3.23*	63
LCFA	0.65	0.05	0.20	59	0.02	0.97	0.34	49
BCFA	0.18	1.09	0.35	58	2.85	2.44	0.66	71
OCFA	2.90	1.51	1.54	68	0.53	1.26	1.63	78
n6 fatty acid	0.36	2.24	2.57	58	0.00	1.11	1.09	48
n3 fatty acid	4.11*	0.43	3.21*	66	0.18	1.57	0.81	51
n6/n3	4.44*	0.09	2.07	58	0.31	1.34	0.19	37
trans fatty acid	<0.01	2.35	0.40	63	1.51	1.21	0.98	44
trans fatty acid C18:1	0.10	2.28	0.35	65	3.67	1.05	1.14	53
Unsaturation index								
C10:1/(C10:0 + C10:1)	0.62	0.64	0.09	63	5.74*	2.02	1.90	65
C14:1/(C14:0 + C14:1)	0.86	0.62	0.07	58	8.50**	1.81	2.16	68
C18:1/(C18:0 + C18:1)	0.04	1.51	1.55	54	4.78*	0.96	0.27	72
RA/(RA + VA)	1.28	0.46	0.72	51	0.09	0.39	0.06	46

[1] Repetability (%) calculated as ratio between $\left(\frac{\sigma^2_r}{\sigma^2_r+\sigma^2_e}\right) \times 100$, where σ^2_r and σ^2_e, are variances attributed to the effect of replicate-animal and error, respectively.

[2] FAs = fatty acids; SFAs = saturated fatty acids; MUFAs = monounsaturated fatty acids; PUFAs = polyunsaturated fatty acids; SCFAs = short-chain fatty acids; MCFAs = medium-chain fatty acids; LCFAs = long-chain fatty acids; BCFAs = branched-chain fatty acids; OCFAs = odd-chain fatty acids; SCFAs included all linear fatty acid from C4:0 to C10:1; MCFA included all linear fatty acid from C11:0 to C16:1; LCFA included all linear fatty acid from C17:0 to C24:1; trans fatty acids included all trans fatty acids; trans fatty acids C18:2 included all trans isomers of C18:1; RA = rumenic acid; VA = vaccenic acid.

[3] BACT = Bacteriological status, expressed in classes (positive; negative).

[4] SCC = somatic cell count, expressed in classes (four classes; class 1 < 50 000 cell/mL; 50 000 cell/mL ≤ class 2 < 200 000 cell/mL; 200 000 cell/mL ≤ class 3 < 400 000 cell/mL; class 4 ≥ 400 000 cell/mL).

[5] BACT × SCC = interaction between the two main effects.

* $P < 0.05$.

** $P < 0.01$.

were present in small amounts (e.g. 18:2 t7, c9 and 20:5 c5, c8, c11, c14, c17).

Associations between somatic cell count and milk fatty acids

At T1, SCC was associated with seven individual FAs, polyunsaturated FA (**PUFA**) and SCFA ($P < 0.05$) (Tables 1 and 2; Fig. 2; Supplementary Figs. S5–S7). Samples with high SCC (≥200 000 cells/mL) had lower proportions of 8:0 and 10:0 ($P < 0.01$) (Supplementary Fig. S5). Individual BCFA generally exhibited the same pattern, with no differences between high SCC and low SCC (except for 14:0 iso), whereas their values were higher in samples with SCC < 50 000 cells/mL than in samples with SCC ranging from 50 000 and 200 000 cells/mL ($P < 0.05$) (Supplementary Fig. S6). In the case of PUFA, samples with high SCC (≥200 000 cells/mL) had higher values than samples with low SCC (<200 000 cells/mL), while SCFA exhibited the opposite pattern (Supplementary Fig. S7).

At T2, SCC was associated with 14 individual FAs (Table 1). In particular, samples with high SCC (≥200 000 cells/mL) had lower proportions of 8:0 ($P < 0.05$), 10:0 ($P < 0.001$), 11:0 ($P < 0.05$), 12:0 ($P < 0.001$), and 13:0 ($P < 0.05$) than samples with low SCC (<200 000 cells/mL) (Supplementary Fig. S5). On the other hand, samples with high SCC had higher values of 15:0 iso ($P < 0.05$), 16:0 iso ($P < 0.01$), and 17:0 ante ($P < 0.05$) (Supplementary Fig. S6). Samples with SCC < 50 000 cells/mL had lower proportions of CLA 18:2 t7, c9 ($P < 0.01$), CLA 18:2 c9,t11 ($P < 0.05$), 20:4 n6 ($P < 0.01$) and 22:5 ($P < 0.001$) than samples with SCC ranging from 50 000 and 200 000 cells/mL (Supplementary Fig. S7).

Associations between the bacteriological status × somatic cell count interaction and milk fatty acids

At T1, the interaction between the bacteriological status and SCC was associated with SCFA and n3-FA ($P < 0.05$) (Table 2). Specifically, among samples with low SCC those that were culture-positive had higher SCFA values than those that were culture-negative ($P < 0.05$). Among the low SCC classes (<200 000 cells/mL), the interaction was however significant only for the class with SCC ranging between 50 000 cells/mL and 200 000 cells /mL ($P < 0.05$). Culture-positive samples had lower proportions of n3 than culture-negative samples for low vs high SCC, and for the SCC class < 50 000 cells/mL ($P < 0.001$) (Fig. 3).

At T2, the BACT × SCC interaction was associated with MCFA (Table 2). Culture-positive samples had higher MCFA compared with culture-negative samples for the low SCC classes (<200 000 cells/mL; $P < 0.001$) and within the low SCC classes for both the SCC class < 50 000 cells/mL ($P < 0.01$) and for the SCC class ranging between 50 000 and 200 000 cells/mL ($P < 0.05$) (Fig. 3).

Discussion

We conducted the first observational study on the associations between natural subclinical IMI and SCC (and their combination) on the detailed milk fatty acid profile at the quarter level in Holstein cows. Despite we had IMI cases from different agents, we were not able to assess potential pathogen-specific effects because of the low prevalence of some pathogens, which did not allow to

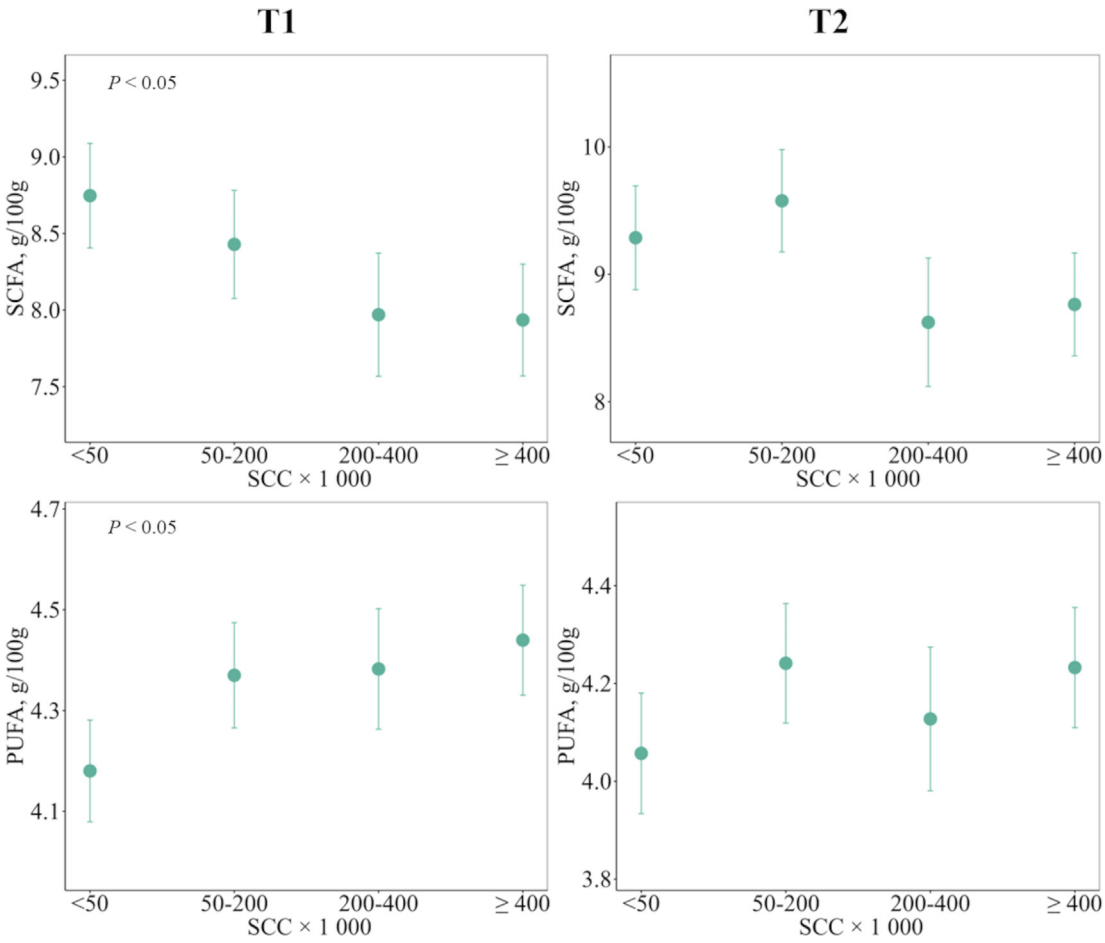

Fig. 2. Least square means and SE of fatty acid groups according to somatic cell count (SCC) classes in Holstein cows. SCFAs: short-chain fatty acids; PUFAs: polyunsaturated fatty acids. T1: sampling time 1 (2 weeks after T0); T2: sampling time 2 (4 weeks after T1). Only significant ($P < 0.05$) traits for at least one sampling time were displayed.

get an adequate data distribution among the levels of all factors included in the mixed model. The results of the bacteriological exam showed that the most prevalent microorganism in the sampled herd was *Streptococcus agalactiae*. Although clinical cases do occur, *Streptococcus agalactiae* is the main cause of chronic and subclinical IMI characterised by high SCC (Djabri et al., 2002). It has been almost eradicated in some North-European countries, but warnings of re-emergence are present. In Italy, only two regions carried out a control programme for this pathogen: Lombardia and Emilia Romagna. As a result, the estimated herd-level prevalence reported in these two regions is around 7–10% (Carra et al., 2021), while data on its prevalence in other Italian regions are not available.

Overall, we observed some changes in the bacteriological status between T1 and T2, which could be due to the pathogens' infection patterns. For instance, *Staphylococcus aureus* can induce chronic infections with intermittent shedding (Sears et al., 1990). In addition, we found stronger associations between IMI combined with SCC and milk FA at T2 than at T1, which could be due to exacerbation of the inflammation (+56% SCS in positive animals; Pegolo et al., 2022). These findings can be explained by the mammary epithelial cell damage associated with high SCC, which, in turn, affect the synthesis of milk components (Turini et al., 2020). Moreover, the patterns of some milk FAs at the two sampling time points did not always agree, further proof that the animals were probably not at the same stage of infection and/or inflammation. Accordingly, changes in bacteriological status between T1 and T2 might also explain these results since we expect some differences in FA profile if a quarter sample was culture-negative at T1 and culture-positive at T2 compared to being culture-positive at both sampling times. The moderate repeatability among individual mammary quarters seemed to support a certain degree of interdependence between them (Paixão et al., 2017).

Intramammary infection combined with somatic cell count and synthesis of fatty acids

Intramammary infection was associated with lower proportions of total and individual SCFA at T2, which can be ascribed to inhibition of the *de novo* synthesis process. Fatty acid synthesis is a complex biological process regulated in the first part by acetyl-CoA carboxylase (**ACC**) and fatty acid synthase (**FASN**). Specifically, ACC is the first enzyme involved in the synthesis of FA from acetyl-CoA, while the action of FASN elongates the chain up to 16 carbon atoms. Li et al. (2021) reported that the expression of *ACC* and *FASN* decreased significantly in the presence of lipopolysaccharide, the main component of the membrane of Gram-negative bacteria. This reduction seems, however, to be independent of the type of pathogen, as it occurred in both *Streptococcus uberis* and *E. coli* infections (Moyes et al., 2009). *In silico* analyses have confirmed the down-regulation of several genes involved in lipid metabolism (including *FASN*) in infected animals compared with healthy animals (Huma et al., 2020).

Some individual SCFA concentrations decreased at increasing SCC, as also found by Turini et al. (2020), which supports an inhibitory effect of SCC also on *de novo* synthesis. Presumably, in the

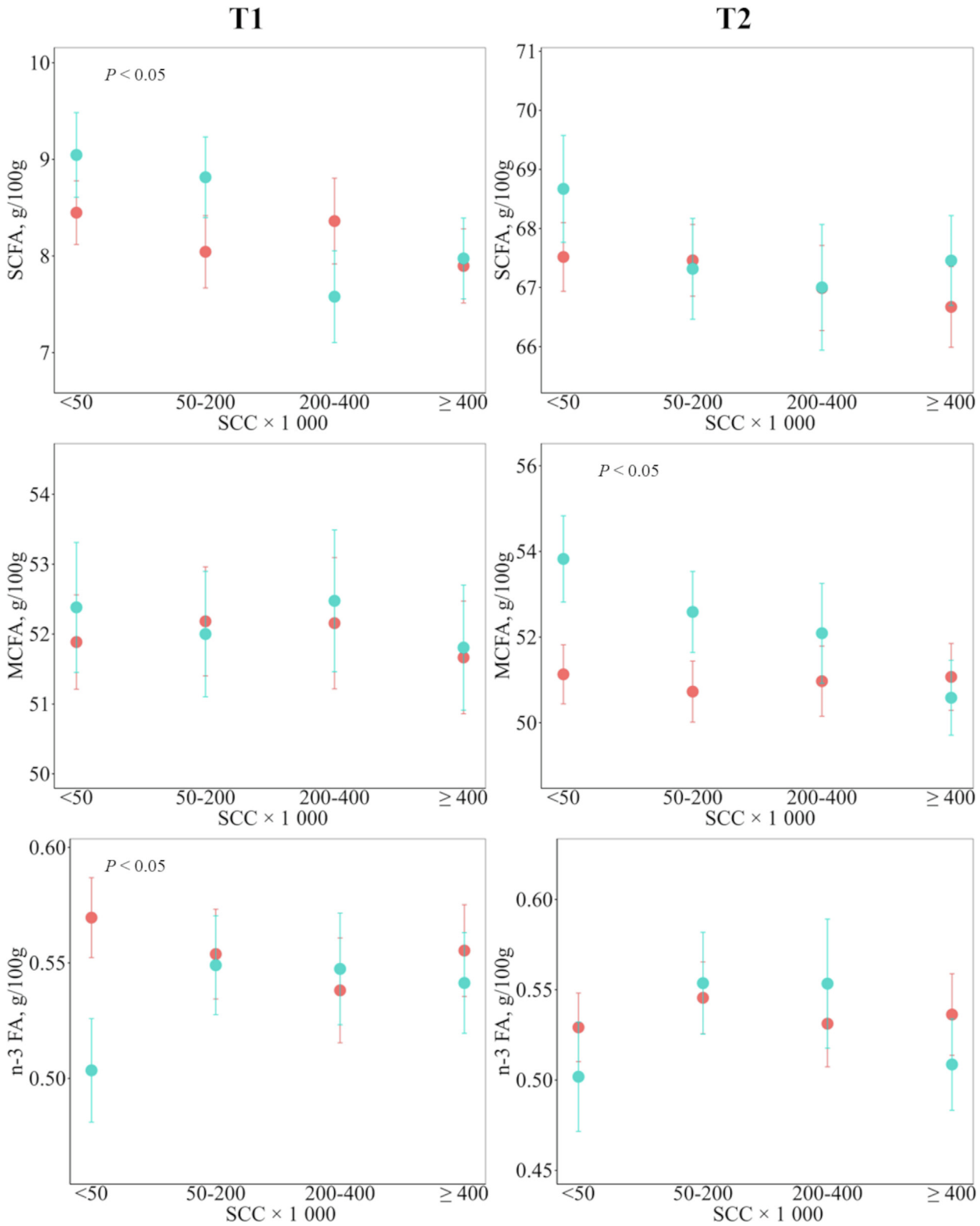

Fig. 3. Least square means and SE of fatty acid groups according to the bacteriological status (BACT) × somatic cell count (SCC) interaction in Holstein cows. SCFAs: short-chain fatty acids; MCFAs: medium-chain fatty acids; n3 FAs: omega-3 fatty acids. T1: sampling time 1 (2 weeks after T0); T2: sampling time 2 (4 weeks after T1). Only significant ($P < 0.05$) traits for at least one sampling time were displayed. Green dots correspond to culture-negative samples, and coral dots correspond to culture-positive animals.

case of severe inflammatory conditions, the *de novo* synthesis process is the first to weaken as a result of the high energy demand to produce new FA (Li et al., 2021), with a consequent increase in the utilisation of FA derived from body reserves or feed. On the other hand, culture-positive quarters had higher total SCFA with respect to the culture-negative samples for the SCC class ranging between 50 000 cells/mL and 200 000 cells /mL ($P < 0.05$). Recent data suggest that SCFAs are an essential source of energy for the structural

proteins of the blood-milk barrier which integrity is critical for the normal physiology of the mammary gland (Akhtar et al., 2022). In addition, SCFA could modulate the innate immune response in the mammary glands by reducing cell adhesion and chemotaxis, and regulating the activity of leukocytes which are inflammatory molecules such as cytokines and/or chemokines (Alva-Murillo et al., 2012). It is possible that the increase in SCFA observed at an early stage of the infection, before the worsening of the inflammatory conditions, is due to an increase in the permeability of the blood-milk barrier. Further studies are, however, needed to support this hypothesis.

Increasing SCC in culture-positive quarters was also associated with a decrease in total MCFA at T2, while in culture-negative quarters, they remained constant across SCC classes despite the presence of a relatively large number of samples with SCC > 200 000 cells/mL (64 out of 218). Inhibition of *FASN* was reported to reduce also MCFA in the mammary gland of goat (Zhu et al., 2014). These results seemed to indicate that MCFA synthesis might be impaired by the infection as inflammation worsened.

Intramammary infection combined with somatic cell count and desaturation of fatty acids

At T2, IMI was associated with a reduction in the 10:1 and 14:1 desaturation indices, which are measures of the efficiency of the desaturation process. This reduction is mainly driven by the decrease in the corresponding MUFA proportions, which is larger than the decrease in the SFA counterparts. On the other hand, despite the significant association between IMI and the 18:1 desaturation index, IMI was not associated with the FA involved in the ratio (18:0 and 18:1 cis 9). This could be related to the different origins of 18:1 cis 9: and 18:0. While 10:1 and 14:1 cis 9 originate exclusively by the desaturation of 10:0 and 14:0 *de novo* synthetised by the mammary gland, 18:0 and 18:1 cis 9 originate by both body fat mobilisation and dietary lipids in a different proportion according to the energy balance of the animal.

The stearoyl-CoA desaturase 1 (**SCD1**) enzyme is rate-limiting for the formation of MUFA by introducing a double bond at position Δ9, controlling key processes in milk fat synthesis. Weller et al. (2019) found that IMI caused by *Streptococcus agalactiae* reduced the expression of the genes involved in lipid metabolism in the liver and udder, including *SCD*. Xu et al. (2016) investigated the mechanism controlling *SCD1* expression in the livers and udders of cows suffering from experimentally induced *E. coli* mastitis, and found that the down-regulation of *SCD* expression in the udder is mediated by the combined enhanced expression of repressive *CCAAT/enhancer-binding protein factors*, and reduced expression of the inducer *sterol regulatory element-binding transcription factor 1*. In addition, Moyes et al. (2009) found down-regulation of *SCD* expression in mammary tissue in response to IMI with *Streptococcus uberis.* Gene network and pathway analyses showed that the *tumour necrosis factor-alpha,* a key gene in immune response during infection, had negative relationships with the genes involved in lipid metabolism, including *SCD*. They also found a putative integration of lipid metabolism and inflammation through the liver X receptor/retinoid X receptor, and proliferator-activated receptor (**PPAR**) signalling pathways. In particular, impaired PPARγ signalling seemed to be associated with down-regulation of lipogenic enzymes such as *SCD* through down-regulation of *sterol regulatory element-binding transcription factor 1* or directly through decreased binding to response elements.

This study has shown for the first time that subclinical IMI combined with SCC is associated with alterations in the milk FA profile3 of individual udder quarters. We have provided phenotypic evidence that IMI combined with SCC mostly affects the *de novo* FA synthesis and desaturation processes within the mammary gland. Furthermore, the moderate repeatability observed among the mammary quarters is an indication that they cannot be considered completely independent units, and that the immune status of a single quarter is likely to influence the overall immune status of the udder. These results contributed to shedding light on the relationships between mammary gland health status and specific pathways involved in milk FA synthesis, as a metabolic response to the infection/inflammation challenge. In addition, they provide useful information for the potential identification of early indicators of infection and/or inflammation in dairy cattle, which could be implemented, together with somatic cell count and other indicators of the mammary gland health status (i.e., lactose, pH, minerals, etc.) at the herd level by the use of high throughput phenotyping techniques (e.g. infrared spectroscopy). Before that however, support is needed from more extensive studies including a larger population and multiple time points.

Supplementary material

Supplementary material to this article can be found online at https://doi.org/10.1016/j.animal.2023.100978.

Ethics approval

This research was approved by the Ethical Committee for the Welfare and Use of Animals (OPBA – Organismo Preposto al Benessere degli Animali) of the Università Cattolica del Sacro Cuore, and by the Italian Ministry of Health (protocol number 510/2019-PR of 19/07/2019).

Data and model availability statement

All the datasets used in this study are available from the authors upon reasonable request.

Declaration of Generative AI and AI-assisted technologies in the writing process

The authors did not use any artificial intelligence-assisted technologies in the writing process.

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A.C.; project administration, **P.A.M.**; funding acquisition, **A.C.** and **P.A.M.** All authors have read and agreed to the published version of the manuscript.

Declaration of interest

The authors declare no conflict of interest.

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