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Heat stress has divergent effects on the milk microbiota of Holstein and Brown Swiss cows.

F. Ceciliani,[1] A. Maggiolino,[2] F. Biscarini,[3] Y. Dadi,[1] L. De Matos, P. Cremonesi,[3] V. Landi,[2] P. De Palo,[2] and C. Lecchi[1]*
[1]Department of Veterinary Medicine and Animal Science, Università degli Studi di Milano, via dell'Università 6, 26900 Lodi, Italy
[2]Department of Veterinary Medicine, Università degli Studi di Bari A. Moro, SP per Casamassima, km 3, 70010 – Valenzano BA
[3]Institute of Agricultural Biology and Biotechnology, National Research Council (CNR), Milan, Italy.

ABSTRACT

Heat stress (**HS**) is one of the pivotal causes of economic losses in dairy industries and affects welfare and performance, but its effect on milk microbiota remains elusive. It is also unclear if and how different breeds may cope with HS in sustaining productive performance. The objectives of this study were to compare a) the performance of 2 dairy breeds, namely Holstein and Brown Swiss, subjected to HS and b) the different effects of HS on the milk microbiota of the 2 breeds in thermal comfort conditions and HS. The study was carried out on 36 dairy cows, 18 per breed. The HS was induced by switching off the cooling system during a natural heat wave for 4 d. Besides the Temperature Humidity Index (THI), the animal stress was confirmed by measuring respiratory frequency and rectal temperature twice daily at 4 a.m. and 3 p.m. The HS differently impacted the 2 breeds. Rectal temperatures were higher in Holstein cows, while no changes in rectal temperature were found in Brown Swiss. Milk yield recording and sampling were performed during the morning milking of d 1 (at 4.00 a.m.) and afternoon milking of d 4 (at 5.00 p.m.). Productive parameters were also different: milk yield, fat-corrected milk, energy-corrected milk, protein and casein content, and renneting parameters were decreased in Holstein but remained unaffected in Brown Swiss. The HS also modified the milk microbiota of the 2 breeds differently. During HS, the Brown Swiss milk microbiota was richer (α diversity) than the Holstein one. Comparing the time points before and during HS within breeds showed that Brown Swiss milk microbiota was less affected by HS than Holstein's. Under the same thermal comfort condition, milk microbiota did not discriminate between Brown Swiss and Holstein. Consistently with α and β diversity, the number of operational taxonomic units (OTUs) at the genus level that changed their abundance during HS was higher in Holstein (74 OTUs) than in Brown Swiss (only 20 OTUs). The most significant changes in abundance affected *Acinetobacter, Chryseobacterium, Cutibacterium, Enterococcus, Lactococcus, Prevotella-9, Serratia,* and *Streptococcus.* In conclusion, the present report confirms and extends previous studies by demonstrating that Brown Swiss cows regulate their body temperature better than the Holstein breed. The relative thermal tolerance to HS compared with Holstein is also confirmed by changes in milk uncultured microbiota, which were more evident in Holstein than in Brown Swiss.
Key Words: Microbiota, milk, Heat stress, cows, Brown Swiss, Holstein

INTRODUCTION

Heat stress (HS) negatively impacts milk production and reproduction performance, immune responses, and overall health and welfare (Becker et al., 2020), resulting in a significant financial burden to the dairy industry. Dairy cows are susceptible during HS according to breed, genetic potential, life stage, management or production system, and nutritional status (Amamou et al., 2019; Cassandro, 2020; Maggiolino et al., 2020). When the average temperature-humidity index (THI) exceeds 68 (approximately 22 °C at 50% relative humidity), overcoming the thermoneutral zone of lactating dairy cows, HS occurs, decreasing feed intake, milk production, and reproductive performance (Bouraoui et al., 2002; Becker et al., 2020; Mishra, 2021). If the HS level increases further, it may become lethal (Burhans et al., 2022). Metabolic heat production of dairy cows increases with milk synthesis, making high-yielding dairy cows extremely susceptible to environmental heat. In contrast, nonlactating cows produce less metabolic heat (West, 2003) and are less vulnerable to environmental heat (Hahn, 1999). Previous

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*Corresponding author: Cristina Lecchi, Address: Via dell'Università 6, Lodi- 26900 – Italy Phone number: +390250334532, e-mail address: cristina.lecchi@unimi.it

The list of standard abbreviations for JDS is available at adsa.org/jds-abbreviations-24. Nonstandard abbreviations are available in the Notes.

investigations reported that Brown Swiss cows showed evidence of heat-stress tolerance when compared with Holstein cows (Correa-Calderon et al., 2004), although more recent studies have highlighted some detrimental effects of HS also in Brown Swiss cows (Maggiolino et al., 2022; Landi et al., 2023a; b). Brown Swiss cows also regulate body temperature more efficiently than Holstein cows (Cuellar et al., 2023).

The microbiota, defined as the assemblage of microorganisms in milk, is crucial in maintaining host homeostasis (Marchesi and Ravel, 2015). The microbiota of bovine milk has been widely investigated (Oikonomou et al., 2014, 2020; Ruegg, 2022). Several studies reported that HS affects the bovine ruminal, intestinal, and fecal microbiota (Correia Sales et al., 2021; Czech et al., 2022; Park et al., 2022; Zhang et al., 2022). Changes in seasons' impact on the microbiota of raw milk have also been investigated (Li et al., 2020b; Guo et al., 2021; Celano et al., 2022). Still, to our knowledge, no specific studies describe HS's impact on the milk's uncultured microbial population. The effect of the core genetic footprint on the milk bacterial population is equally poorly addressed. Recent studies have identified the microbiota diversity in milk samples from Holstein cows with different estimated breeding values for feed efficiency and resilience to mastitis (Tarrah et al., 2022) and from 2 breeds, Holstein Friesian and Rendena (Cremonesi et al., 2018). How the milk microbiota from different breeds changes during HS is unknown. The present study aimed to close these gaps and describe the effects of a natural heat wave developed in field conditions on the milk microbiota from Holstein and Brown Swiss breeds reared in the same commercial farm in the South of Italy. The further aim was to determine whether HS-induced changes in performance might differ between the 2 breeds..

MATERIALS AND METHODS

Experimental design

The trial was carried out during the summer of 2022 and has been approved by the Ethical Committee for experiments with animals of the Department of Veterinary Medicine of the University of Bari (Approval Number 05/2022). The experimental model was developed to evaluate the consequences of sudden temperature increases, such as during a heat wave. The study was carried out on 40 multiparous mid-lactating Holstein (n = 20, days in milk (DIM) 106 ± 9.12; BCS: 2.41 ± 0.12; mean ± SD) and Brown Swiss (n = 20, DIM 102 ± 7.30; BCS: 2.67 ± 0.15; mean ± SD) dairy cows balanced for parity and days in milk (DIM - between 80 and 160 DIM), reared in the same commercial farm located in the Apulian Region in the South of Italy. The freeware software Lenth, R. V. (2006–9) was used to determine the minimum sample size (Retrieved 16 April 2022, from http://www.stat.uiowa.edu/~rlenth/Power). The experimental design considered the 2 breeds and the 2 times, and for sample size calculation, the value of α was set to 0.05 and β to 0.20 for a power of 0.90. The expected difference was set at 9.5. The milk yield and its SD (10.2) (Franzoi et al., 2020) were the outcomes considered for sample size calculation. The sample size obtained was 18 cows for each group, but we involved 20 animals, considering the possibility of excluding some during the trial. The animals were clinically healthy, the somatic cell content of milk was $<100 \times 10^3$ cells per mL, and the microbiological analysis for pathogen search was negative. Four data loggers (Hobo Pro series Temp probes, Onset Computer Corp., Pocasset, MA, USA) recorded environmental temperature and humidity every 5 min across the trial period. Two data loggers were located in the cubicle lying area (at the height of the animal's head), and the other 2 were in different positions in the feeding area. The devices were placed at the height of animals' heads. The lactating cows' barn was equipped with coolers, automatic sprinklers in the feeding area, and roof fans in the cubicle resting area. The heat wave was induced by switching off the cooling system for 4 consecutive days and then re-activating it. The Temperature Humidity Index (THI) was calculated according to the formula previously applied (Landi et al., 2023b)

$$THI = (1.8 \times AT + 32) - (0.55 - 0.55 \times RH) \times [(1.8 \times AT + 32) - 58],$$

where AT is the environmental temperature expressed in degrees Celsius, the term (1.8 × AT + 32) represents the conversion of temperature data in degrees Fahrenheit. RH is the relative humidity as a fraction of the unit. The mean THI obtained by the 4 dataloggers was considered.

Heat stress measurement: respiratory rate and rectal temperature

The heat stress measurement was defined by changes in physiological data, including respiratory rate and rectal temperature. Physiological data were recorded at 4.00 a.m. on day one, the coolest moment before HS, and considered the thermal comfort condition (TC), and at 3.00 p.m. on d 4, the hottest moment of the day after 4 HS days and regarded as the HS condition. Data on THI TC and HS are presented in Figure 1.

Respiratory rate (RR) measurements were visually taken by trained personnel observing the movement of the animal's rib cage for 60 s. Rectal temperatures (RT)

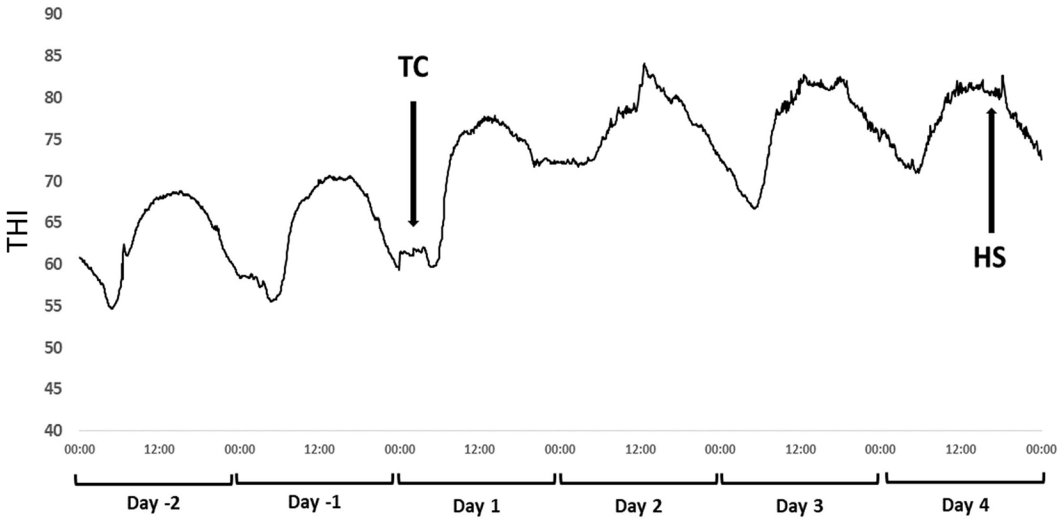

Figure 1. THI changes during the induced HS experimental condition. The arrows point out the end of the Thermal Comfort condition (TC) and the end of the Heat Stress condition (HS), which includes also the frametime for the performance record s and sample collection. THI = Temperature-Humidity Index, TC = Thermal Comfort, HS = Heat stress.

were measured by a digital rectal thermometer (Gima, Gessate, Milan, Italy).

Milk sampling and analysis

Milk yield recording and sampling were performed during the morning milking of d 1 (at 4.00 a.m.) and the afternoon milking of d 4 (at 5.00 p.m.). The milk parlor was not cooled. From each cow, 100mL of milk were collected in sterile containers from the 4 quarters, and 2% 2-Bromo-2-nitro-1,2-propanediol were added as a preservative, refrigerated at 4°C, transferred to the laboratory, and analyzed within 2 h with near-infrared spectroscopy for fat, protein, lactose, dry matter, urea, BHB and fatty acid profile (unsaturated fatty acids, UFA; monounsaturated fatty acids, MUFA; polyunsaturated fatty acids. PUFA; saturated fatty acids, SFA) (ISO9622/IDF141: 2013). From these data, fat-corrected milk (FCM) yield, standardized at 4% fat, was calculated for each test-day record according to the following formula (Maggiolino et al., 2020):

$$4\%\ \text{FCM} = 0.4 \times \text{milk} + 15 \times \text{fat}$$

The energy-corrected milk (ECM) yield was calculated according to the formula previously reported (Yan et al., 2011):

$$\text{ECM} = \text{milk} \times [0.25 + (0.122 \times \%\text{fat}) + (0.077 \times \%\text{protein})]$$

Milk coagulation properties were determined via Formagraph (Foss Analytics) using commercial rennet as previously described (Franzoi et al., 2020).

DNA extraction, library preparation, and sequencing

The uncultured microbiome was determined on 11 animals per breed and 2 time points. The animals whose milk was included in the present study were those with highest RT. We changed the text accordingly and we refer not to the study, that is at its second round of evaluation in JDS.The bacterial DNA was extracted by combining a chaotropic agent, guanidium thiocyanate, with silica particles to increase bacterial cell lysis and nuclease inactivation, as described previously (Cremonesi et al., 2021). The method was suitable for healthy whole milk samples with a low bacterial load. The DNA concentration was assessed using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The isolated DNA was stored at −20°C until use. For library preparation, bacterial DNA was amplified using the primers described in the literature (Caporaso et al., 2011b), which target the V3-V4 hypervariable regions of the 16S rRNA gene, as suggested by the Illumina protocol. All PCR amplifications were performed in 25 µL volumes per sample. A total of 12.5 µL of Phusion High-Fidelity Master Mix 2 × (ThermoFisher Scientific, Walthem, MA, USA) and 0.2 µL of each primer (100 µM) were added to 2 µL of genomic DNA (5 ng/µL). Blank controls (i.e., no DNA template added to the reaction) were also performed. A first amplification step was conducted in an Applied Biosystem 2700 thermal cycler (ThermoFisher

Scientific). Samples were denatured at 98°C for 30 s, followed by 25 cycles with a denaturing step at 98°C for 30 s, annealing at 56°C for 1 min and extension at 72°C for 1 min, with a final extension at 72°C for 7 min. Amplicons were cleaned with Agencourt AMPure XP (Beckman, Coulter Brea, CA, USA), and libraries were prepared following the 16S Metagenomic Sequencing Library Preparation Protocol (Illumina, San Diego, CA, USA). The libraries obtained were quantified by Real-Time PCR with KAPA Library Quantification Kits (Kapa Biosystems, Inc., MA, USA), pooled in equimolar proportion and sequenced in one MiSeq (Illumina) run with 2 × 250-base paired-end reads.

Microbiota Characterization of milk samples

After demultiplexing, forward and reverse paired-end reads from 16S rRNA-gene sequencing were assembled into single reads using the C++ program SeqPrep (John, 2011). After joining, reads were filtered for quality based on: (i) maximum of 3 consecutive low-quality base calls (Phred <19) allowed; (ii) fraction of consecutive high-quality base calls (Phred >19) in a read over total read length ≥0.75; (iii) no "N"-labeled bases (missing/uncalled) allowed. Reads that did not match all the above criteria were excluded. All remaining reads were combined in a single FASTA file to identify and quantify OTUs (operational taxonomic units). Reads were aligned against the SILVA closed reference sequence collection release 132, with 97% cluster identity (Quast et al., 2013; Yilmaz et al., 2014) applying the CD-HIT clustering algorithm (Li & Godzik 2006). Taxonomies were identified (domain, phylum, class, order, family, and genus), and the resulting OTU table was filtered by removing OTUs with less than 10 total counts in fewer than 2 samples. Before subsequent analysis, OTU counts were normalized for uneven sequencing depth by cumulative sum scaling CSS (Paulson et al., 2013).

The milk microbiota diversity was assessed within (α diversity) and across (β diversity) samples. Besides the number of observed OTUs directly counted from the OTU table, within-sample microbial richness, diversity, and evenness were estimated using the Chao1, ACE, Shannon, Simpson, Fisher α, Simpson E and equitability (Pielou J a.k.a. Shannon evenness) (Fisher et al., 1943; Shannon, 1948; Simpson, 1949; Bray and Curtis, 1957; Chao, 1984; Chao and Lee, 1992; Smith and Wilson, 1996). The across-sample microbiota diversity was quantified by calculating Bray–Curtis dissimilarities (Bray and Curtis, 1957). Among-groups (before and during HS) and pairwise Bray–Curtis dissimilarities were evaluated non-parametrically using the permutational ANOVA (999 permutations, PERMANOVA)(Anderson, 2001). Details on calculating the α and β diversity indices can be found in Biscarini et al. S2 Appendix (Biscarini et al., 2018).

Random Forest

A random forest (RF) (Breiman, 2001) model for binary classification was developed to predict thermal conditions (HS vs TC) based on CSS-normalized OTU counts (at the genus level) of milk samples. 5-fold cross-validation was used to fine-tune the number of variables randomly sampled in each tree, from the range [-5, +5] around the square root of the number of variables "p" —microbial taxa— in the data (from sqrt(p)-5 to sqrt(p)+5, step size = 1). As for the number of trees in the RF model and the minimum size of terminal nodes, the default values of 500 and 10, respectively, were chosen. The Gini index ($G = k = 1\ \hat{p}\ mk\ (1 - \hat{p}\ mk)$, where $\hat{p}$ mk is the proportion of samples in the m th belonging to class k) was used to decide on splits (the smaller, the better: if all $\hat{p}$ mk 's are close to zero or one then G is small, indicating node purity: the node mainly contains samples of the same class). From the final RF model after hyperparameter-tuning, classifications were obtained by majority vote over the B = 500 classification trees; this process was repeated 100 times using 100 bootstrapped replicates of the data to measure the accuracy of classification on out-of-bag (OOB) observations. From the final RF model, variable importance was also obtained based on the total decrease in Gini index from splitting on the variable, which was averaged over all trees. The RF model analysis was carried out on all cows together and on Holstein and Brown Swiss cows separately.

Statistical analysis

The physiological and milk parameters data were analyzed with Shapiro–Wilk tests for normality distribution and revealed no deviation from normality, so parametric statistics were used. Physiological and milk parameters were analyzed by generalized linear mixed models using PROC MIXED with fixed effects of breed, thermal condition, and their interaction according to the following model:

$$Yijkl = \mu + \alpha i + Bj + Tk + (B \times T)jk + \varepsilon ijkl$$

where Yijk represents the investigated parameter as a dependent variable, μ is the overall mean; αi is the ith dairy cow random effect (i = 1,…18), Bj is the effect of the jth breed (j = 1, 2), Tk is the effect of the Tth thermal condition (k = 1, 2), (B × T)jk is the binary interaction the jth breed and the Tth Thermal condition (jk = 1,…4) and εijk is the error term. A pairwise comparison was performed using the Bonferroni test. Significance was

set at $P < 0.05$; the results are expressed as least squares means and standard error of the means. All the analyses were performed using SAS software (SAS, 2018). Significance was set at $P < 0.05$.

The differences between TC and HS in terms of α diversity indices, phylum relative abundances, and OTU counts at the genus levels were analyzed with the following 2 models: i) across breeds (all cows), y_ijk = mu + breed_k + time point_j + e_ijk (equation (2)); ii) within the breed (Holstein and Brown Swiss cows separately), y_ij = mu + time point-j + e_ij (equation (3)).

In both models, y_i(jk) refers to α diversity indices values, phylum relative abundance, or OTU counts for sample i from breed k (where applicable) at time point j (TC or HS); breed_k and time point_j refer to the effects of breed and thermal condition respectively; e_i(jk) are the model residuals.

Softwares

Milk microbiota sequencing data were processed with the Quantitative Insights into Microbial Ecology (QIIME) open-source bioinformatics pipeline for microbiome analysis v. 1.9 (Caporaso et al., 2011a). More details on the command lines used to process 16S rRNA-gene sequence data can be found in Biscarini et al. S1 Appendix (Biscarini et al., 2018). The Abundance-based Coverage Estimator (ACE) index and sample-based rarefaction were estimated using a custom Python script (https://github.com/filippob/Rare-OTUs-ACE.git). The Random Forest models were developed using the R package ranger (Wright and Ziegler, 2017). Plots were generated using the ggplot2 R package (Wickam, 2016). Additional data handling and statistical analysis were performed with the R environment for statistical computing (R Core Team 2023).

RESULTS

Physiological responses to heat stress

Results about respiration rate and rectal temperature are reported in Figure 2. Hyperthermia affected the respiration rate in both breeds ($P < 0.0001$), showing higher values during HS than TC. The increase in temperature equally induced HS in both breeds ($P > 0.05$). The rectal temperature increased with HS in both breeds ($P < 0.0001$). Although there were no differences between the investigated breeds in TC conditions ($P > 0.05$), HS increased rectal temperature with higher values in Holstein compared with Brown Swiss dairy cows ($P < 0.01$).

Heat stress-related changes in milk yield and composition

The results of the milk parameters are reported in Table 1. Milk yield, FCM, and ECM were affected by HS ($P < 0.05$) only in Holstein cows, showing lower values in HS compared with TC. No differences were observed in Brown Swiss cows and between breeds ($P > 0.05$). Protein and casein were affected by breed ($P < 0.01$), with lower values in Holstein cows both in TC and HS conditions. The fat and fatty acid profiles did not change due to breed, thermal condition, or their interaction ($P > 0.05$). Only breed affected renneting parameters ($P < 0.0001$). Holstein always showed lower curd firmness, higher clotting velocity, and renneting time values ($P < 0.0001$).

Changes in the uncultured bacterial population

The first set of results compared the general microbial profile of milk collected from healthy Holstein and Brown Swiss in TC. The second set investigated how HS may affect the milk microbiota profile of the 2 breeds.

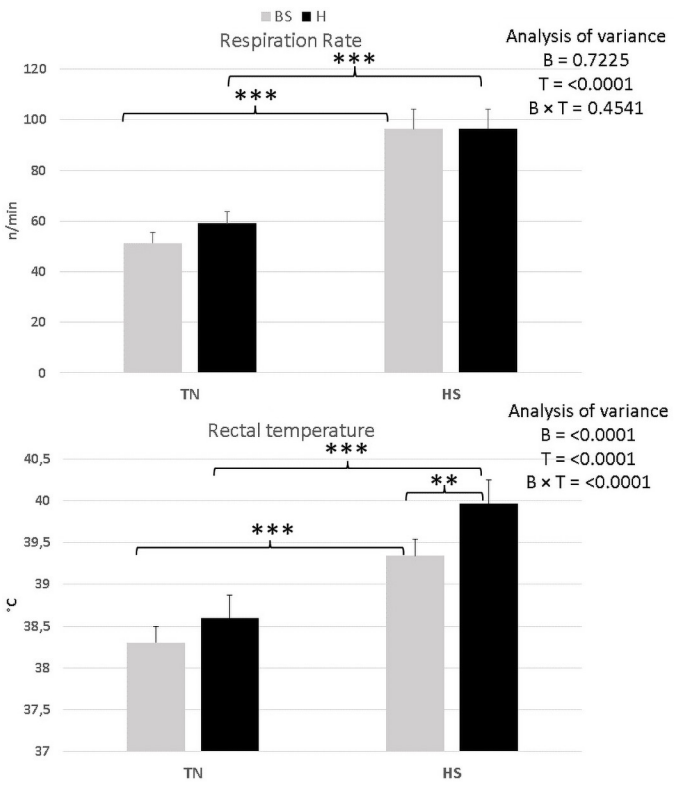

Figure 2. The effects of HS on respiration rate and rectal temperature. The figure presents the changes induced by HS on the respiration rate and rectal temperature. HS = Heat Stress, TC = Thermal Comfort condition. B = Breed; T = Thermal condition. Grey Bars (BS): Brown Swiss, Black bars (H): Holstein. ** = $P < 0.01$; *** = $P < 0.0001$.

Table 1. Description of milk yield, fat-corrected milk (FCM), energy-corrected milk (ECM) fat, protein, casein, and lactose percentage, saturated fatty acids (SFA), unsaturated fatty acids (UFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), Curd firmness, Clotting time and Renneting time of the BS = Brown Swiss and H = Holstein cows. B = Breed; TC = Thermal comfort condition; HS = Heat stress; T = Thermal condition

Parameter	Breed	TC	HS	SEM	B[1]	T[2]	B × T
Milk Yield (kg)	BS	35.22	32.58	1.36	0.5966	0.0230	0.4698
	H	36.52^{a}	31.02^{b}				
FCM yield (kg)	BS	42.47	38.29	1.91	0.6622	0.0354	0.5655
	H	42.73^{a}	35.49^{b}				
ECM yield (kg)	BS	38.63	35.49	1.78	0.6897	0.0322	0.5420
	H	38.93^{a}	32.64^{b}				
Fat (%)	BS	4.79	4.82	0.11	0.0345	0.6166	0.6339
	H	4.59	4.46				
Protein (%)	BS	3.46^{x}	3.48^{X}	0.07	<0.0001	0.6489	0.8452
	H	3.16^{y}	3.15^{Y}				
Casein (%)	BS	2.70^{x}	2.69^{X}	0.07	<0.0001	0.8466	0.7332
	H	2.43^{y}	2.46^{Y}				
Lactose (%)	BS	4.83	4.94	0.02	0.6810	0.4251	0.5120
	H	4.84	4.83				
SFA (%)	BS	3.25	3.18	0.08	0.0225	0.6935	0.3663
	H	3.14	2.99				
UFA (%)	BS	1.47	1.45	0.04	0.0401	0.8744	0.4789
	H	1.39	1.37				
MUFA (%)	BS	1.31	1.32	0.04	0.2373	0.7644	0.4332
	H	1.29	1.27				
PUFA (%)	BS	0.14	0.12	0.004	0.0128	0.5941	0.8245
	H	0.13	0.12				
Curd firmness (mm)	BS	24.21^{X}	23.18^{X}	1.35	<0.0001	0.7654	0.7114
	H	13.35^{Y}	13.70^{Y}				
Clotting velocity (min)	BS	7.76^{X}	7.82^{X}	0.24	<0.0001	0.7945	0.4662
	H	9.86^{Y}	9.58^{Y}				
Renneting time (min)	BS	28.25^{X}	29.19^{X}	0.83	<0.0001	0.8841	0.3927
	H	33.15^{Y}	32.98^{Y}				

Different letters in the same line show statistical differences between thermal conditions in the same breed: a, b = $P < 0.05$.

Different letters in the same row show statistical differences between breeds in the same thermal condition: X, Y = $P < 0.01$; x, y = $P < 0.05$.

a) Discriminant analysis and clustering of samples (Alpha and Beta Diversity) The α diversity indices, reported as averages, in the milk microbiota of the 2 breeds at TC and HS are shown in Table 2. The α diversity analysis indicated that the microbiota under HS was richer than under TC (all richness and diversity indices) and less homogeneous (reduced evenness indices: equitability and Simpson E). These results were driven by divergent responses in Brown Swiss cows, where the richness and diversity of the milk microbiota increased, and Holstein cows, where microbial richness and diversity decreased. However, the differences in Brown Swiss cows were all significant, while none were significant in Holstein cows (Figure 3). In the aggregate, 3 metrics out of 8 (ace $P = 0.04$; equitability $P = 0.01$; simpson_e $P = 0.005$) were significantly different during HS, even though all other index differences were close to the significance threshold (p-values between 0.06 and 0.10). In detail, the milk microbiota of Brown Swiss was richer under HS than under TC (e.g., 776.72 vs. 583.818 observed OTUs, $P = 0.002$; 1150.8 vs 718.5 Chao1, $P = 0.001$; 1161 vs 731.1 ACE, $P = 0.001$; 461.13 vs 319.59 Fisher's α, $P = 0.002$; 9.167 vs. 8.801 Shannon index, $P = 0.004$), while no significant differences were found in Holstein comparing TC and HS. Comparing the 2 breeds under the same thermal conditions, under TC, the microbiota of Holstein was richer than Brown Swiss (725.455 vs. 583.82 observed OTUs, $P = 0.04$; 1016.41 vs. 718.48 Chao1, $P = 0.02$; 1008.67 vs. 731.05 ACE, $P = 0.02$), while during HS, the microbiota of Brown Swiss was richer than Holstein (1150.78 vs 900.19 Chao1, $P = 0.027$; 1161.01 vs 935.19 ACE, $P = 0.04$).

Beta diversity analysis was based on measuring Bray-Curtis dissimilarities. The first 2 dimensions from the multidimensional scaling of the Bray-Curtis dissimilarity matrix, clustering samples by thermal conditions, breeds, and thermal conditions x breeds, are illustrated in Figure 4. Beta diversity analysis showed clustering by thermal condition (TC vs. HS), both overall (Figure 4A) ($P = 0.0009$) and within breeds (Holstein group $P = 0.001$ and Figure 4B; Brown Swiss group $P = 0.009$ and Figure 4C). Milk microbiota could not discriminate between Brown

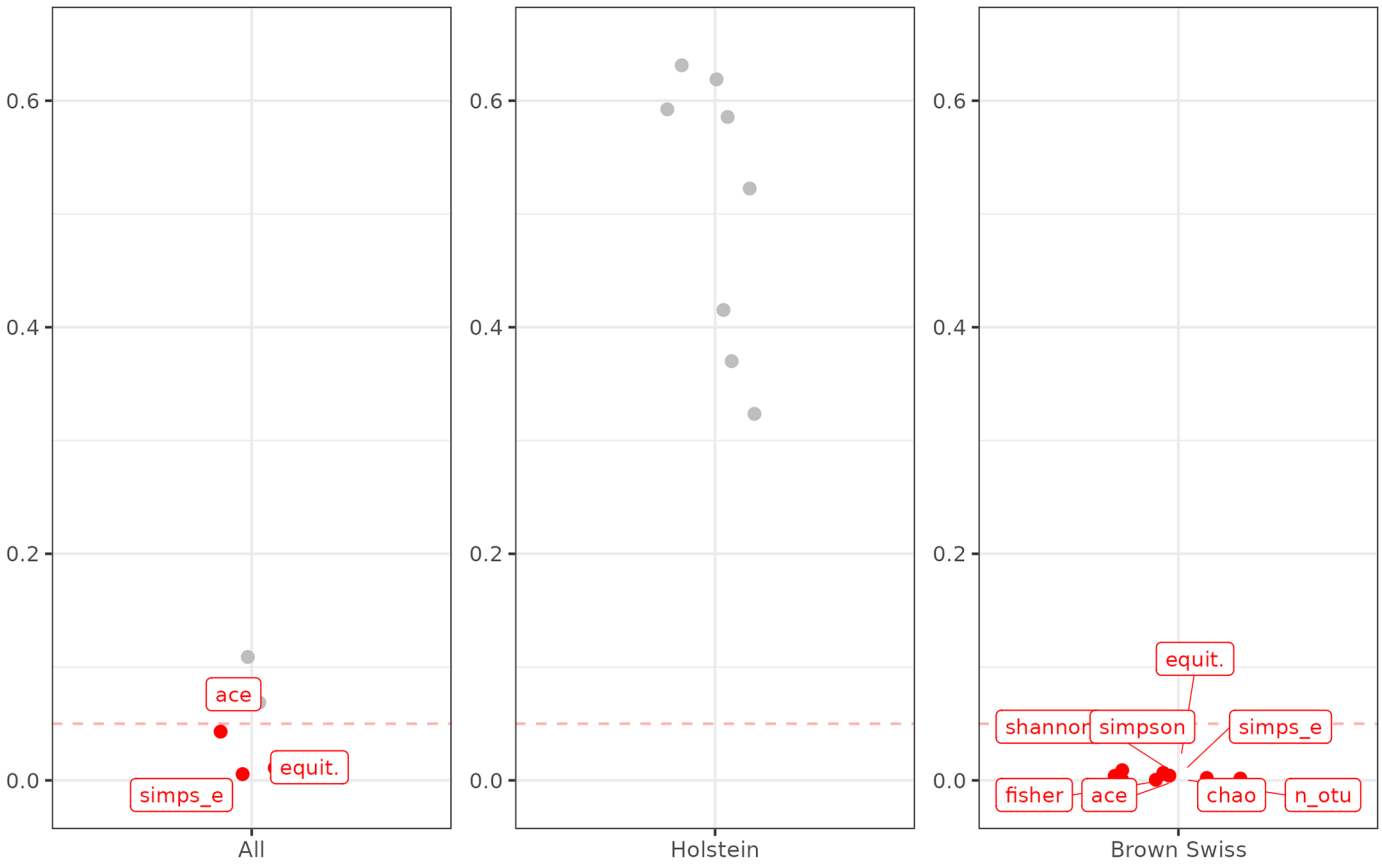

Figure 3. Alpha diversity indexes in Thermal Comfort condition (TC) and Heat Stress (HS). BS: Brown Swiss; H: Holstein. The significant indices are pointed out.

Swiss and Holstein under the same thermal condition, at both TC and HS, as the difference between breeds from PERMANOVA was not significant ($P = 0.51$).

The composition of the milk microbiota at the phylum level before and during HS is shown in Figure 5 for all cows combined (A) and for the 2 breeds separately ((B) Holstein, (C) Brown Swiss).

The milk microbiota was mainly composed of Proteobacteria (TC 80.1%; HS 73.8%; $P = 0.0089$), Firmicutes (HS 18.1%; TC 11.69%; $P = 0.0002$), Bacteroidetes (HS 5.3%; TC 4.5%; $P = 0.2448$) and Actinobacteria (TC 2.9%; HS 2.1%; $P = 0.1322$). Proteobacteria were significantly more abundant at TC, while Firmicutes were significantly more abundant during HS.

Within breeds, significant changes in the relative abundance of phyla between TC and HS included Firmicutes (13.1% vs. 19.4%, $P = 0.02$) and Actinobacteria (3.6% vs. 1.7%, $P = 0.013$) in Holsteins, and Proteobacteria (83.4% vs 74%, $P = 0.0022$), Firmicutes (10.3% vs 16.9%, $P = 0.0026$) and Bacteroidetes (3.46% vs 5.82%, $P = 0.0121$) in Brown Swiss cows.

At the genus level, the microbiota of milk during TC and HS was characterized by significant differences in the average abundance of 109 genera ($P < 0.05$) (Supple-

Table 2. Average values of the α diversity indices measured in Thermal Comfort conditions (TC) and during heat stress (HS) in all cows (BS + H) and separately in Brown Swiss (BS) and Holstein (H) cows

breed	time point	N	chao1	ace	fisher_alpha	observed_otus	Shannon	Simpson	equitability	simpson_e
All	before HS	22	867.45	869.86	366.15	654.64	8.938	0.997	0.961	0.614
All	during HS	22	1025.49	1048.10	425.90	734.68	9.092	0.998	0.957	0.58
BS	before HS	11	718.48	731.05	319.59	583.82	8.801	0.997	0.962	0.628
BS	during HS	11	1150.78	1161.01	461.13	776.73	9.167	0.998	0.956	0.574
H	before HS	11	1016.41	1008.67	412.72	725.46	9.075	0.998	0.959	0.6
H	during HS	11	900.19	935.19	390.67	692.64	9.018	0.997	0.957	0.586

mental Table S1 - Figure 6). Among those with the lowest P value were alphabetically, *Chryseobacterium, Cutibacterium, Enterococcus, Lactococcus, Prevotella-9, Serratia, Streptococcus*, in Holstein cows, and *Acinetobacter, Chryseobacterium, Cutibacterium, Enterococcus, Lactococcus, Prevotella-9,* and *Streptococcus* in Brown Swiss cows. Interestingly, *Streptococcus, Enterococcus, Chryseobacterium,* and *Lactococcus* increased in both species, whereas the behavior of *Prevotella 9* is divergent (decreases in Holstein and increases in Brown Swiss).

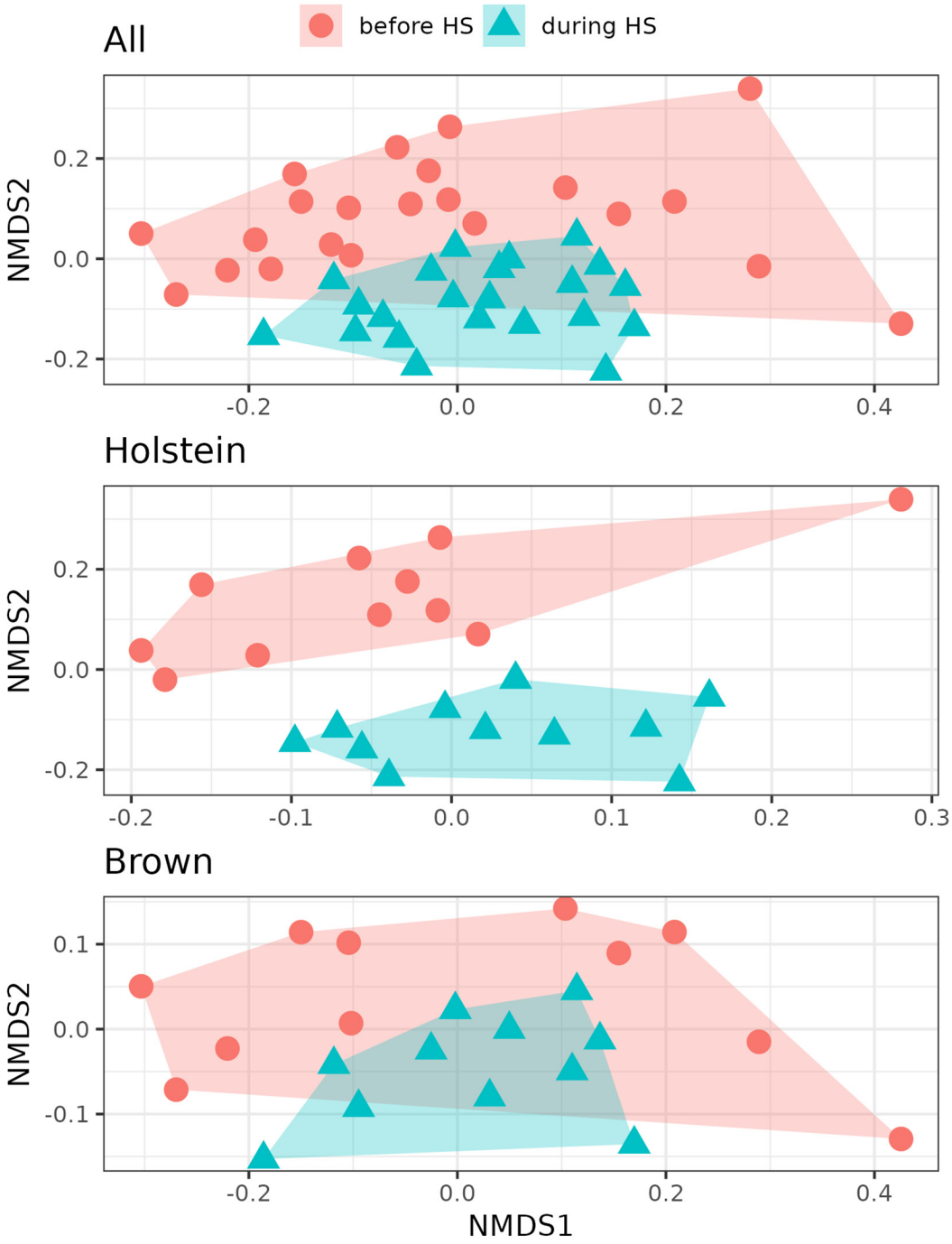

Figure 4. Beta diversity Indexes in Thermal Comfort condition (TC) and Heat Stress (HS). Beta diversity according to thermal comfort condition (TC() vs Heat Stress (HS) in the 2 breeds together (A) and separately in the Holstein (B) and the Brown Swiss (C) groups. Non-metric Multidimensional Scaling (NMDS).

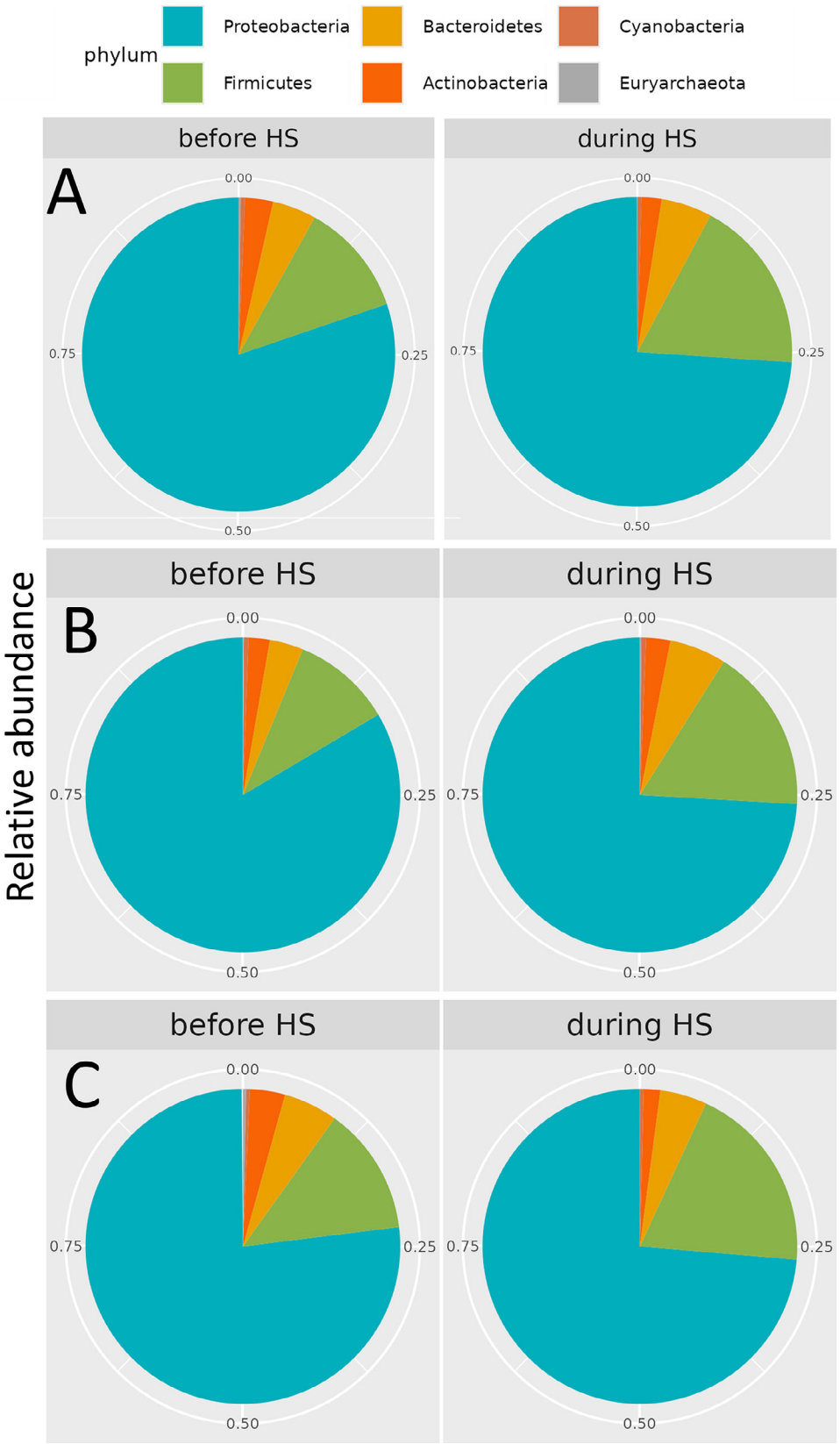

Figure 5. relative abundance of phyla in the milk microbiota, before (TC) and after heat stress (HS), in all cows (A) and in the Holstein (B) and Brown Swiss (C) breeds separately. Phyla with relative abundance lower than 0.1% weren't considered.

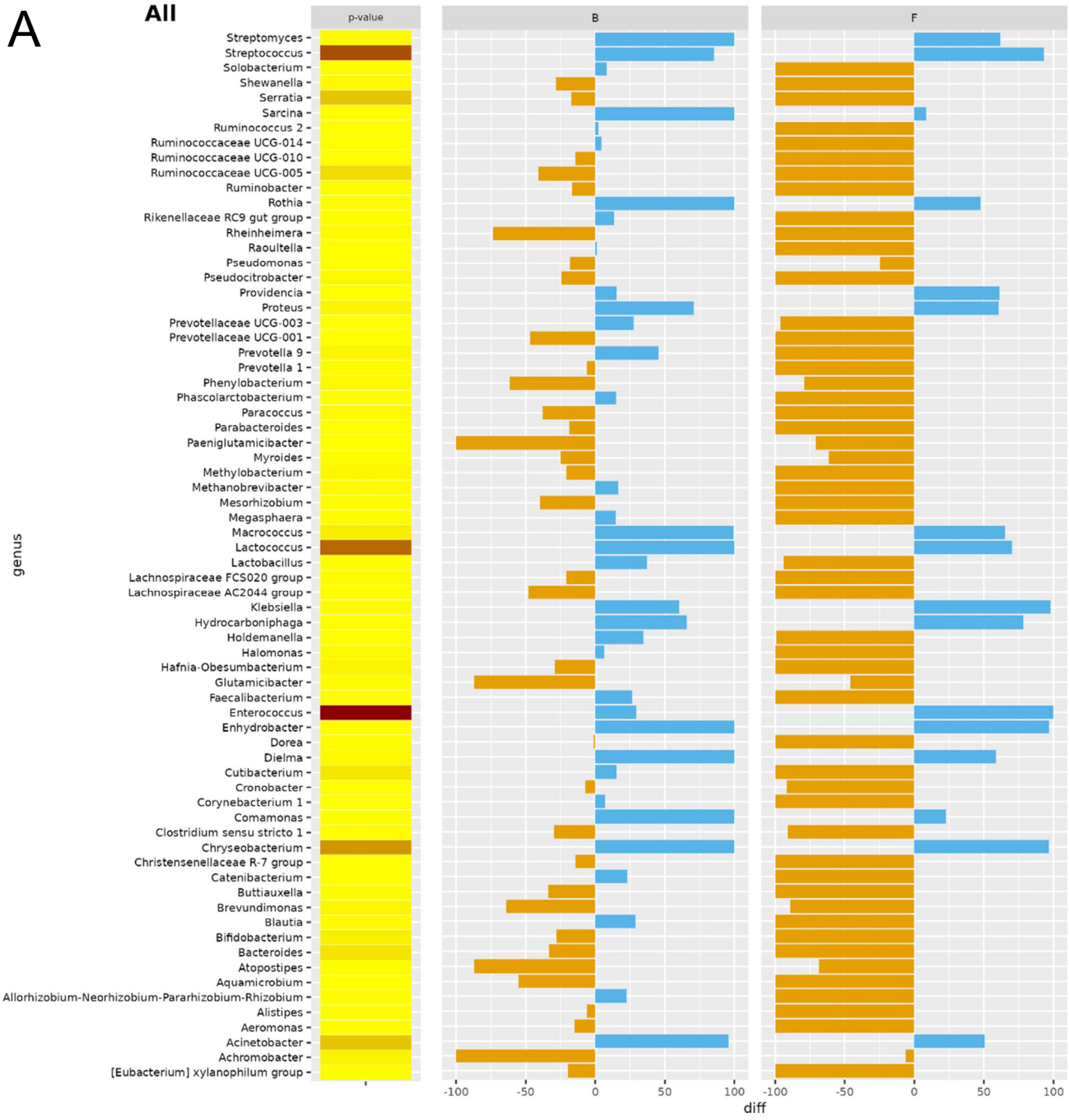

Figure 6. – Significantly different OTUs (at the genus level) in terms of abundance between TC and HS.Panel A: all cows together (equation 2); Panel B: results from the analysis carried out separately for Holstein and Brown Swiss cows (Panel C) (equation 3). Significance is visually represented by colors from yellow ($P = 0.05$) todarker red tones (smaller p-values). Differential abundance between TC (left, orange bars) and HS (right,blue bars) is rescaled to be between 0 and 100 (for each genus separately). Figure 6b – Significantly different OTUs (at the genus level) in terms of abundance between TC and HS. Panela A: all cows together (equation 2); Panel B: results lts from the analysis carried out separately for Holstein and PBrown Swiss cows (Panel B) (equation 3). Significance is visually represented by colors from yellow ($P = 0.05$) to darker red tones (smaller p-values). Differential abundance between TC (left, orange bars) and HS (right, blue bars) is rescaled to be between 0 and 100 (for each genus separately). Figure 6c – Significantly different OTUs (at the genus level) in terms of abundance between TC and HS.Panela A: all cows together (equation 2); Panel B: results from the analysis carried out separately for Holstein and Brown Swiss cows (Panel C) (equation 3). Significance is visually represented by colors from yellow ($P = 0.05$) todarker red tones (smaller p-values). Differential abundance between TC (left, orange bars) and HS (right,blue bars) is rescaled to be between 0 and 100 (for each genus separately).

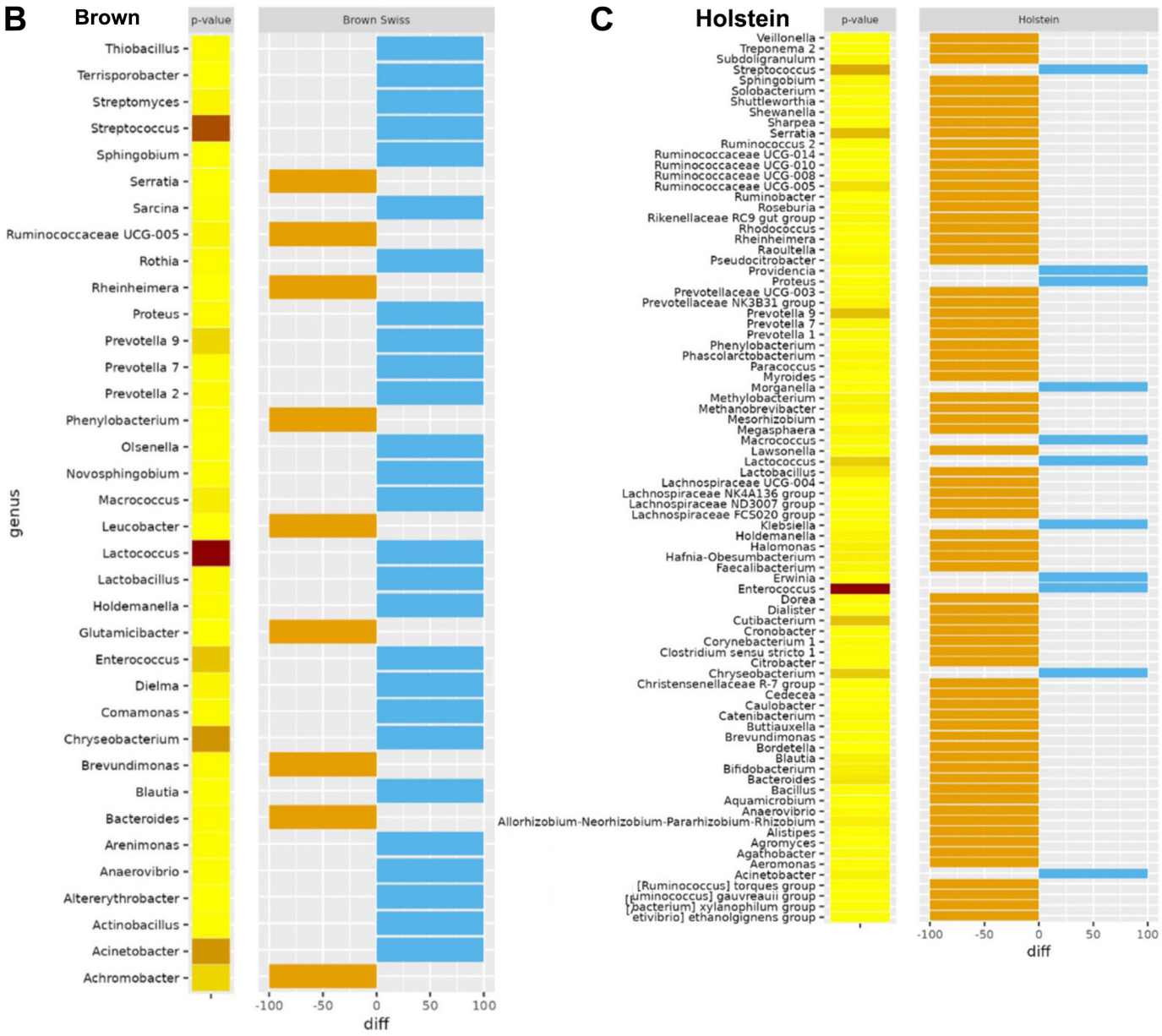

Figure 6 (Continued). – Significantly different OTUs (at the genus level) in terms of abundance between TC and HS.Panel A: all cows together (equation 2); Panel B: results from the analysis carried out separately for Holstein and Brown Swiss cows (Panel C) (equation 3). Significance is visually represented by colors from yellow ($P = 0.05$) todarker red tones (smaller p-values). Differential abundance between TC (left, orange bars) and HS (right,blue bars) is rescaled to be between 0 and 100 (for each genus separately). Figure 6b – Significantly different OTUs (at the genus level) in terms of abundance between TC and HS. Panela A: all cows together (equation 2); Panel B: results lts from the analysis carried out separately for Holstein and PBrown Swiss cows (Panel B) (equation 3). Significance is visually represented by colors from yellow ($P = 0.05$) to darker red tones (smaller p-values). Differential abundance between TC (left, orange bars) and HS (right, blue bars) is rescaled to be between 0 and 100 (for each genus separately). Figure 6c – Significantly different OTUs (at the genus level) in terms of abundance between TC and HS.Panela A: all cows together (equation 2); Panel B: results from the analysis carried out separately for Holstein and Brown Swiss cows (Panel C) (equation 3). Significance is visually represented by colors from yellow ($P = 0.05$) todarker red tones (smaller p-values). Differential abundance between TC (left, orange bars) and HS (right,blue bars) is rescaled to be between 0 and 100 (for each genus separately).

Remarkably, the number of OTUs at the genus level that changes in Holstein (74 OTUs changes in abundance) was higher than those in Brown Swiss (20 OTUs changes in abundance). Moreover, comparing the 2 breeds, in Holstein, most of the changes related to HS induced a decrease in the OTU abundance. In contrast, in Brown Swiss, most of the changes at the OTU level increased in abundance.

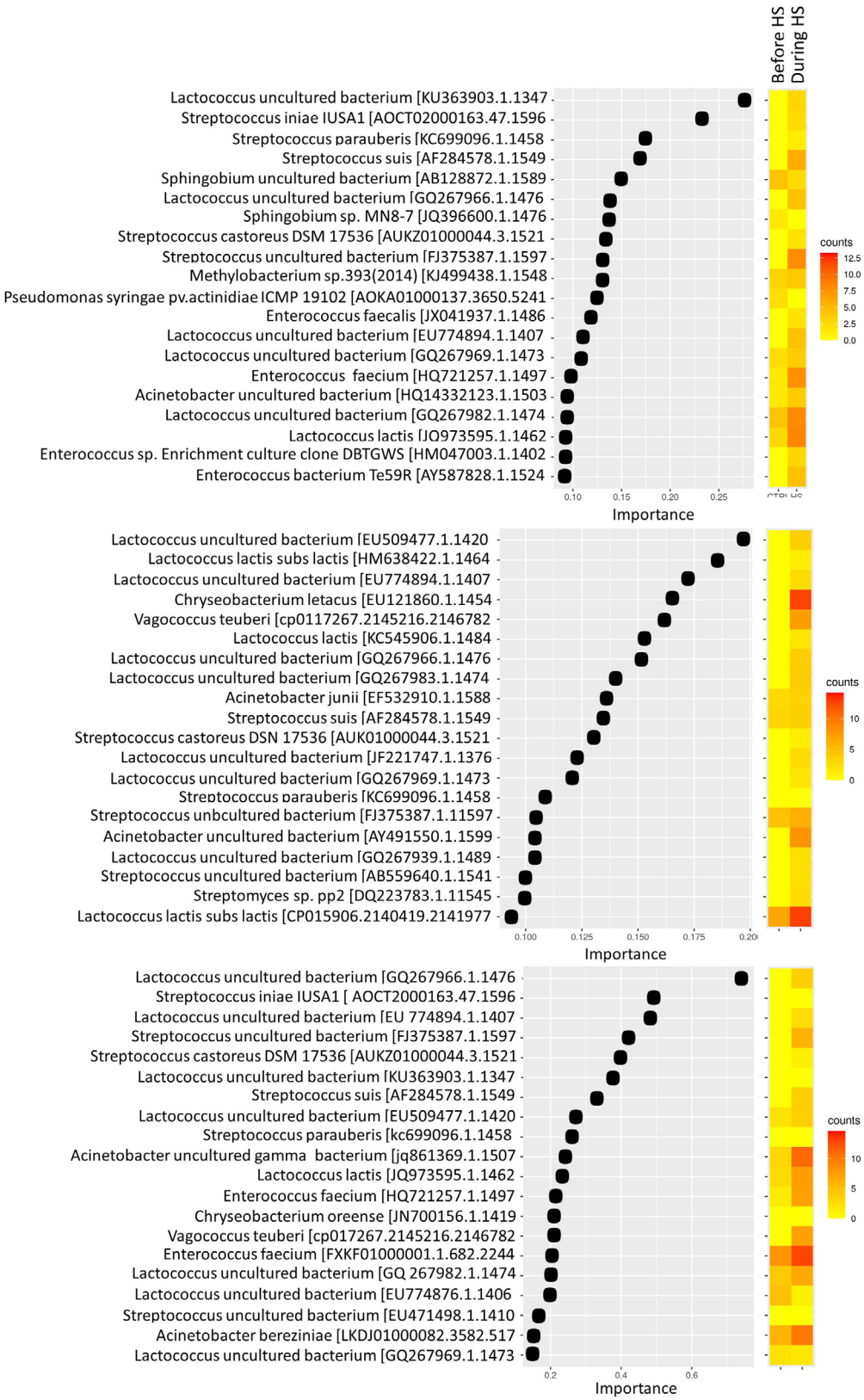

Figure 7. Random Forest prediction. Top 20 OTUs (genus level) ranked according to decreasing variable importance from the random Forest (RF) models: A) all cows together; B) Holstein cows; C) Brown Swiss cows. Variable importance was determined based on the average decrease of the Gini index attributable to each OTU.

Random Forest predictive analysis

In the last part of the study, the microbiota composition was used to predict which group (HS or TC) samples belong to using a Random Forest (RF) model. The RF model achieved an OOB predictive accuracy of 92.3% (ROC AUC = 0.979) when all cows were used together in the analysis (average values from 100 bootstrapped replicates). In Holstein and Brown Swiss, prediction accuracy was 89.8% and 79.9%, respectively. Figure 7 presents the top 20 variables (OTUs at genus level) obtained from the importance of the RF variable. Given the high prediction accuracy of RF models, these results provide evidence that uncultured genera belonging to the Lactococcus phyla perform better in predicting the effect of HS on milk microbiota.

DISCUSSION

One of the most relevant challenges facing animal dairy production is improving milk quality and quantity, increasing efficiency, without compromising animal welfare. Considering dairy cows, high-production animals are subjected to a more significant influence of the climatic environment and HS, particularly those raised under tropical conditions, due to high air temperatures and relative humidity. The association between hot and humid climates (with high THI) and low productive performance of dairy cows represents a limiting factor (Collier et al., 2008; Martello et al., 2010). Heat stress causes changes in the homeostasis processes and can be identified by physiological parameters like rectal temperature and respiration rate measurements, considered the gold standard for HS detection in dairy cows (Berman et al., 1985; Dikmen and Hansen, 2009; Wang et al., 2018; McArthur, 1987; McGovern and Bruce, 2000; Martello et al., 2010). Our results showed that the animals of both breeds suffered HS, showing higher rectal temperature and respiration rate: both increased, confirming the positive correlation between these 2 parameters, with higher rectal temperature observed in Holstein cows. Rectal temperature increase signifies a lack of thermal balance, heat loss mechanisms become insufficient to maintain the equilibrium, and cows increase water intake to replace evaporative losses (Mohammed and Johnson, 1985). A rise in rectal temperature of 1°C or less is enough to reduce DMI and milk yield in dairy cows. However, body temperature is usually maintained by the thermoregulatory system within 1°C of its normal under ambient conditions that do not impose severe HS (Rejeb et al., 2016). By studying the correlation between milk production and rectal temperature and THI, the study reported that, in general, when rectal temperature values are above 38.2°C, there is a decrease in milk yield of 1.1 kg/day for each point of degree. The difference in rectal temperature rising between breeds can be due to different heat tolerance, as reflected by the ability to maintain normal body temperature (Srikandakumar and Johnson, 2004), as well as the start to decline in milk yield with an increase in THI, this is suggestive of higher metabolic heat production in Holstein cows. Our results reported a decrease in milk yield, FCM, and ECM. However, this was significant only in Holstein, confirming the differences in heat tolerance between Holstein and Brown Swiss (Cuellar et al., 2023). Many authors reported a correlation between HS response in dairy cows and their milk production, considering high-producing cows more susceptible to heat ambient (Carabaño et al., 2014). Cows with higher production have high body temperatures (Yano et al., 2014), and their threshold temperature for the onset of HS can be lower by up to 5°C (Li et al., 2020a).

Although milk yield and the FCM and ECM were affected by HS, milk fat concentration and fatty acid profile, protein, casein, lactose concentration, and coagulation parameters, no variation was observed due to HS. Heat Stress can alter coagulation properties in sheep's milk (Sevi and Caroprese, 2012), possibly due to the use of fat and nitrogen reserves to supply energy through gluconeogenesis or to increased milk pH due to high amounts of CO_2 dissipated via the panting (Amaral-Phillips et al., 1993) as well as plasmin activity (Bianchi et al., 2004). The 4 d of induced HS in this trial could be considered insufficient time to affect these parameters. The only pointed-out differences were between the 2 breeds. The low protein content, poor coagulation properties, low frequency of the κ-casein, and low casein content of Holstein breed milk are consistent with previous findings (De Marchi et al., 2007, 2008).

The second part of the study focused on the impact of HS on milk microbiota. Heat stress exerted a different effect on the milk microbiome of the 2 breeds. In thermal comfort conditions, the Holstein microbiome was richer than Brown Swiss. The relationship was inverted in HS, the Brown Swiss microbiome being richer than the Holstein cows. Furthermore, HS increased the richness of bacteria species only in Brown Swiss, as shown by all the indexes tested. This result is interesting, given that the microbiota richness is related to the development of mastitis (Ma et al., 2021). Heat stress also had a different impact on the overall taxonomic composition of the milk of the 2 breeds, as shown by the Bray Curtiss index, which is regarded as the most reliable and sensitive to describe the differences between groups (Kers and Saccenti, 2021): although changes were significant in both breeds, modifications related to HS were much more evident in Brown Swiss, suggesting that this breed is less affected by HS, at least for what regards the milk microbiota. The changes at the taxonomic level also confirmed

the relative resistance to changes related to the increase in temperature: the number of OTUs at the genus level that changed their abundance during HS (74 OTUs) in Holstein was much more than in Brown Swiss (only 20 OTUs).

Interestingly, the significant changes in abundance affected the same genera in Holstein and Brown Swiss, namely *Chryseobacterium, Cutibacterium, Enterococcus, Lactococcus, Prevotella-9, and Streptococcus.* The *Acinetobacter* abundance changed in Brown Swiss only, and *Serratia* only changed in Holstein. Although all the OTUs found have already been described in the milk microbiota, this is the first report associating their abundance changes with HS in milk. Among the OTUs that changed their abundance due to HS, *Streptococcus, Prveotella_9,* and *Acinetobacter* have been reported as HS sensitive, although in the rumen, which is an entirely different environmental scenario (Feng et al., 2023). Also, in the rumen, changes in *Streptococcus* due to temperature increase were reported (Uyeno et al., 2010; Zhao et al., 2019). The genus *Streptococcus* comprises several species. In milk, it has been reported that the effect of environmental temperature increases the abundance of some *Streptococcus* species (*Streptococcus uberis*) and meanwhile decreases the abundance of others (*Streptococcus agalactiae*) (Brown et al., 1977). This result is significant because HS may also increase *Streptococcus* adherence and internalization to mammary gland epithelium (Almeida et al., 2018), thus increasing mammary gland susceptibility during HS. Increased abundance of some genera, like *Lactococcus*, is also associated with increased fermentation and milk spoilage (Quigley et al., 2013). Remarkably, a predictive random forest approach identified *Lactococcus* as those genera that could predict if milk has been produced from HS cows, speculating that these OTUs may be regarded as markers of HS. Indeed, this speculative hypothesis has to be further experimentally demonstrated.

This study has some limitations. The study's experimental design was planned to maximize and highlight differences in the thermotolerance capability of the animals and between breeds. With this background, the first sampling was in the morning milking, after which the fans were switched off, and the second one was in the afternoon milking after 4 d. Although, to the best of our knowledge, no significant change in the microbial content is reported between the different microbiota in milk from morning and afternoon milking, it is known that there are circadian changes involving metabolites, hormones, and cytokines (Teng et al., 2021). Therefore, we may not rule out that some of the changes reported between the breeds were also related to the circadian rhythm. This hypothesis has to be experimentally verified.

In conclusion, the present report confirms and extends previous studies by showing that Brown Swiss cows regulate their body temperature better than the Holstein breed. Consistently, some productive parameters, like protein content and milk coagulation properties, were less affected in Brown Swiss than in Holstein. Changes in uncultured milk microbiota also confirm the relative thermal tolerance to HS of the Brown Swiss as compared with Holstein, the changes of which were more evident in Holstein as compared with Brown Swiss. Further studies are undergoing at proteome, lipidome, and metabolome levels to define the underlying mechanisms of mammary gland adaptation to HS and how it can affect milk production and quality at specific breed levels.

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ORCIDS

F. Ceciliani https://orcid.org/0000-0002-9199-5757
A. Maggiolino https://orcid.org/0000-0001-7128-8556
Y. Dadi https://orcid.org/0009-0000-8923-0395
L. De Matos https://orcid.org/0000-0002-5468-8459
V. Landi https://orcid.org/0000-0003-1385-8439
P. De Palo https://orcid.org/0000-0002-5612-1691
C. Lecchi https://orcid.org/0000-0002-7262-1696