1 **Influence of milk processing temperature on growth performance, nitrogen**
2 **retention, and hindgut's inflammatory status and bacterial populations in a calf**
3 **model**

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5

6 # Supplementary file

7 *Supplementary methodologies*

8 *Method for extracting RNA and assessing gene expression and bacterial counts*

9 One microgram of RNA was reverse-transcribed to cDNA using IScript cDNA synthesis kit
10 (Bio-Rad, California, USA) following manufacturer's instructions. The RNA purity was
11 assessed by a NanoDrop instrument (ND-1000 spectrophotometer; NanoDrop Technologies,
12 Rockland, DE) at 260, 280 and 230 nm, obtaining 260/280 ratios between 1.9-2.0 and
13 260/230 between 2.0-2.2. The relative quantification of gene expression was performed by
14 qPCR using *b-actin* (*actb*) as a housekeeping gene. The qPCR conditions for each set of primers
15 were individually optimized (Table S1).The specificity of the amplification was evaluated
16 by single band identification at the expected molecular weight in 0.8% DNA agarose gels
17 and a single peak in the melting curve. The efficiency was calculated by amplifying serial
18 1/10 dilutions of each gene amplicon. A standard curve of Ct vs log concentration was plotted
19 to obtain the efficiency, which was calculated using the formula $10^{1/slope}$, with an acceptable
20 range of 1.8-2.2. A total reaction volume of 20 µL was used, containing 50 ng of cDNA, 10
21 µL of SYBER green fluorescent (Bio-Rad, California, USA), and the optimized primer
22 concentration for each gene (Table 1). The qPCR reactions were cycled as follows: an initial
23 denaturing step of 10 min at 95°C, followed by 40 cycles of 10 s at 95°C, 15 s at optimized
24 annealing temperature for each gene, 30 s at 72°C and a final extension of 10 min at 72°C.
25 For bacteria quantification DNA was extracted from faecal samples using the RBB+C
26 method, which employs bead beating in the presence of high concentrations of sodium
27 dodecyl sulphate, salt, and EDTA, and subsequent DNA purification with QIAamp

28 (QIAGEN, California, USA) columns (Yu & Morrison, 2004). The samples were submitted to

29 qPCR procedures similar to those for gene expression analyses.

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31 **References**

32 **Yu Z & Morrison M** 2004 Improved extraction of PCR-quality community DNA from

33 digesta and fecal samples. *BioTechniques* **36**: 808–812

34 **Table S1.** Sequence, annealing temperature (At), concentration (μM), amplicon size (bp) of the forward and reverse primers used for qPCR

Item	Forward sequence	Reverse sequence	Annealing temperature, ºC	Concentration, μM	Size, bp
TNFα	AACAGCCCTCTGGTTCAAAC	TCTTGATGGCAGACAGGATG	60	0.5	296
TGF-β	TGAGCCAGAGGCGGACTACT	TGCCGTATTCCACCATTAGCA	60	0.5	61
IL-10	ACTTTAAGGGTTACCTGGGTTG	GAAAGCGATGACAGCGCCGC	57	0.5	170
IL-8	TTGAGAGTGGGCCACACTGTG	TGCACCCACTTTTCCTTGG	55	0.5	93
Occludin	ATCAACCCCGGTGCCGGAAG	GTGGTCTTGCTCTGCCCGCC	57	0.5	162
ß-defensin	GGTCACAAGTGGCAGAGGAT	TGGTTGAAGAACTTCAGGGC	60	0.25	152
TLR4	TCAGAAACCTCCGCTACCTTG	TTCTGAAAAGAGTTGCCTGCC	55	0.5	117
Claudin-4	CATGATCGTGGCCGGCGTG	AGGGCTTGTCGTTGCGGG	62	0.125	226
IL-1β	TGGGAGATGGAAACATCCAG	TTTATTGACTGCACGGGTGC	50	0.3125	232
Lactobacillus	GCAGCAGTAGGGAATCTTCCA	GCATTYCACCGCTACACATG	60	0.25	348
Gram positive	GAYGACGTCAARTCMTCATGC	AGGAGGTGATCCAACCGCA	50	0.5	168
Gram negative	AYGACGTCAAGTCMTCATGG	AGGAGGTGATCCAACCGCA	65	0.5	352
Faecalibacterium	CAGCAGCCGCGGTAAA	CTACCTCTGCACTACTCAAGAAA	53	0.5	151
Bifidobacterium	GGGTGGTAATGCCGGATG	CCACCGTTACACCGGGAA	58	0.5	510

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37 **Table S2.** Milk content of protein, fat, lactose of the milks fed in this study

	Raw	Pasteurized	UHT	SE	P-value
Protein, %	3.13	3.18	3.15	0.04	0.76
Fat, %	3.80	3.78	3.79	0.02	0.73
Lactose, %	4.84	4.82	4.86	0.01	0.56

38 [a,b] Values with uncommon superscripts differ at $P < 0.05$.

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