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

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This research communication describes a study aimed at evaluating the effects of heat treatment of milk on growth performance, N retention, and hindgut's inflammatory status and bacterial populations using young dairy calves as a model. Twenty-one Holstein calves were randomly allocated to one of three treatments: raw milk (RM), pasteurised milk (PAST), or UHT milk (UHT). Calves were submitted to a N balance study, and a biopsy from the distal colon and a faecal sample were obtained from 5 animals per treatment to determine expression of several genes and potential changes in the hindgut's bacterial population. Milk furosine content was 33-fold greater in UHT than in RM and PAST milks. Calves receiving RM grew more than those fed UHT, and urinary N excretion was greatest in calves fed UHT. Quantification of *Lactobacillus* was lower in calves consuming PAST or UHT, and Gram negative bacteria were greater in UHT than in PAST calves. The expression of IL-8 in the hindgut's mucosa was lowest and that of IL-10 tended to be lowest in RM calves, and expression of claudin-4 tended to be greatest in UHT calves. In conclusion, the nutritional value of UHT-treated milk may be hampered because it compromises growth and increases N excretion in young calves and may have deleterious effects on the gut's bacterial population and inflammation status.

**Keywords:** Intestinal microbiota, milk processing, nutrient absorption.

The nutritional value of milk proteins is generally recognised as excellent. To extend the shelf-life of milk, the industry applies different heat treatments. The two most common heat treatments are pasteurisation (typically at 72 °C for 15 s), which allows milk to be preserved at 4 °C for relatively long periods (one or two weeks), and sterilisation by ultra-high-temperature (UHT; 135–150 °C for 2–6 s), which renders the milk distributable at ambient temperature for months. Milk has a number of functional or bioactive compounds (fatty acids, bioactive peptides, enzymes, vitamins, immunoglobulins etc), and it is commonly assumed that the nutritional benefits associated with the consumption of raw milk are unaffected after pasteurisation or UHT treatments. Nevertheless, pasteurisation destroys about 10 to 30% of bovine milk immunoglobulin activity, and UHT treatment destroys the majority of the immune activity of milk (Claeys et al. 2014). Also, during heat treatment the ε-amino groups of lysine residues react with reducing sugars, such as lactose, and form Amadori products such as lactuloselysine, lysinoalanine and carboxymehtyl-lysine (Al-Saadi et al. 2012). Last, some soluble proteins may also be altered during heat treatment as they undergo denaturation, which ultimately may lead to protein aggregation and reduced digestibility (Nicorescu et al. 2009).

To our knowledge, only a few *in vivo* studies (Povoa & Moraes-Santos, 1997; Lacroix et al. 2006; Lacroix et al. 2008) have addressed the potential consequences of heat treatment on the nutritional impact of milk. Lacroix et al. (2008) reported a reduction in N retention in individuals consuming UHT milk compared with those consuming microfiltered milk (unheated). On the other hand, the gut microbiota is known to have a role in the development of the immune system, and may modulate the host's metabolism and energy balance (Oikonomou et al. 2013). Thus, the objective of this study was to evaluate the effects of heat treatment of milk on growth performance, N retention, intestinal bacterial population, and intestinal inflammatory status using young dairy calves as a model. Calves were chosen as a model because of their ability to consume large amounts of milk and because their metabolism has many similarities to that of humans (Allen et al. 2005).

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## Materials and methods

All animals and procedures used herein were managed under the approval and following the guidelines of the Animal Care Committee of IRTA.

### *Animals and treatments*

Twenty one healthy Holstein male calves (initial body weight = 42·5 ± 4·71 kg; initial age = 4·3 ± 1·38 d) were acquired from the same dairy herd, transported (trip duration <2 h) to IRTA facilities, and randomly allocated to one of three treatments: Raw milk (RM), pasteurised milk (PAST), or UHT milk (UHT). While at their farm of origin, all calves received 3 l of colostrum within 6 h after birth, followed by a second dose of colostrum of 2 l within 12 h. Then, calves were fed 2 l of raw milk twice daily until they were transported to IRTA. Once enroled in the study, calves were offered the same milk feeding program: 6 l/d from the beginning of the study to 12 d, and 8 l/d from day 13 to the end of the study at day 22, distributed in two equal portions at 0800 and 1600 h. Milk was collected every two days from the bulk tank (saleable milk) of the same farm, located 3 km from IRTA, and either stored raw at 4 °C (RM), or pasteurised at 72 °C for 15 s (PAST) and then stored at 4 °C, or treated at ultra-high temperature (142 °C) for 6 s (UHT) and then also stored at 4 °C. All milk batches were stored for a maximum of 3 d. Calves were kept in individual hutches (2 × 2·2 $m^2$) and bedded daily with sawdust. During the first 18 d of study, each calf was offered 50 g/d of barley straw to satisfy their potential needs to consume solid feed while minimising potential confounding effects of solid feed intake with the study measures. Also, all animals had *ad libitum* access to fresh water throughout the study.

### *Measures*

Milk protein and fat contents from every batch were determined by Fourier transform infrared spectroscopy. Also, milk samples from all treatments were obtained for subsequent determination of furosine and carboxymethyl-lysine as proxies of biological value of the different milks.

Calves were weighed weekly using an electronic scale, and milk refusals recorded daily. From 19 to 22 d of study, all calves were moved to adjacent individual hutches (2 × 2·2 m) floored with plastic slats to allow total urine collection (by gravity) and conduct a nitrogen balance study. During these 3 d, total faecal and urine collection took place. The plastic bottom of each hutch (where urine was collected) had 200 ml of 5N HCl to prevent microbial growth and avoid N loses through volatilisation. Total daily urine output for each calf was weighed and mixed thoroughly, and then a 5% aliquot was collected for subsequent analysis. Total faecal collection was performed by plastic bags that were attached to the rear of each calf as described by Terré et al. (2007). Plastic bags were collected 3 times per day and frozen at −20 °C.

On day 20 of study, a biopsy from the distal colon (about 40 cm from the anus) was obtained from 5 animals (randomly chosen) per treatment using an endoscope (Olympus GIF-N180, Barcelona, Spain). Biopsies were rapidly rinsed in PBS and incubated and suspended on RNAlater (Ambion, Austin, TX) overnight at 4 °C, then RNAlater was removed and tissue was frozen at −80 °C until subsequent RNA extraction and determination of expression of genes encoding for $TNF_{\alpha}$, and $TGF_{\beta}$, IL-10, IL-8, occludin, β-defensin, TLR-4, claudin-4, and IL-1β.

On day 22 of study, a faecal sample from each calf was collected from the rectum and immediately frozen at −20 °C to later determine relative abundance of *Lactobacillus*, total Gram positive, and total Gram negative bacteria using qPCR. It has been previously reported that there is a high correlation between bacterial populations in faeces and in the caecum of ruminants (Mao et al. 2015).

### *Chemical and molecular determinations*

Milk protein content was determined following the procedure 992·15 for total protein content (AOAC, 2000) and using a N-to-protein conversion factor of 6·38. Milk fat content was determined using the method described by IDF (2010) in the method ISO 1211:2010. The milk content of furosine was determined following the ISO 18329:2004 method (IDF, 2004). Carboxymethyl-lysine was analysed by liquid chromatography coupled to a tandem mass spectrometry and stable isotope dilution ($D_2$-CML) after an acid digestion of milk samples and a reduction with $NaBH_4$.

Faecal dry matter content was determined by oven drying (24 h at 103 °C). Faeces fat content was quantified as ether extract determined following the AOAC (2000) method (920·39) using petroleum ether for distillation instead of diethyl ether (AOAC, 2000). Faecal N content was determined following the AOAC (2000) method (988·05) adapted for an automatic distiller Kjeldhal (Kjeltec Auto 1030 Analyser, Foss, Denmark) and using $CuSO_4$/Se as catalyst instead of $CuSO_4$/$TiO_2$. Total N in urine samples was determined using the macro-Kjeldahl procedure following the AOAC (2000) method 976·05.

For gene expression analyses, RNA was extracted from colon wall tissues using Trizol® (Invitrogen, Madrid, Spain). Methods for gene expression are provided in the Supplementary file. Likewise, for bacteria quantification, total microbial DNA was extracted as described in the Supplementary file. The resulting Ct values were used to calculate the expression of selected genes by relative quantification using a reference gene (beta-actin) and a calibrator. A sample, corresponding to an animal in the RM treatment, was randomly chosen as the calibrator following the method proposed by Pfaf (2004).

### *Calculations and statistical analyses*

Sample size was determined based on the primary outcome variable: urinary N excretion. A power analysis with an 80%

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**Table 1.** Intake, growth, digestion and N retention of calves as affected by the type of heat treatment applied to the milk consumed

| | Milk type | | | | *P*-value | | |
|---|---|---|---|---|---|---|---|
| | Raw | Pasteurised | UHT | SE | T | W | T × W |
| Milk intake (l/d) | 6·65[a] | 6·45[a] | 5·91[b] | 0·22 | 0·02 | <0·001 | 0·18 |
| Straw intake (g/d) | 13·9[a] | 7·7[b] | 6·9[b] | 1·59 | 0·01 | <0·001 | 0·01 |
| Initial BW (kg) | 42·1 | 41·0 | 43·7 | 1·79 | 0·56 | — | — |
| Average daily gain (g/d) | 720[a] | 694[ab] | 488[b] | 79·6 | <0·01 | <0·001 | 0·28 |
| Dry matter digestion (%) | 92·2 | 93·0 | 92·6 | 0·94 | 0·82 | | |
| Fat digestion (%) | 95·6 | 95·4 | 96·1 | 0·90 | 0·89 | | |
| Crude protein digestion (%) | 93·1 | 93·1 | 92·0 | 0·77 | 0·72 | | |
| Urinary N excretion (g/d) | 2·56[b] | 2·36[b] | 4·11[a] | 0·23 | 0·003 | | |
| Nitrogen retention (%) | 91·1 | 91·3 | 88·0 | 0·78 | 0·17 | | |

T: Effect of treatment; W: Effect of week; T × W: effect of the interaction between treatment and week
[a,b]Values with uncommon superscripts differ at $P < 0·05$

power, a type I error of 0·05, and assuming a standard deviation (SD) of 0·4 g/d revealed that 7 animals would be needed to detect a 30% difference in urinary N excretion among 3 treatment means. Nitrogen retention was calculated as the percentage of N intake that was not excreted in urine and faces. Digestibility of dry matter (DM), crude protein (CP), and fat were calculated as the quotient between the daily intake of each of these nutrients and the amount of each of these nutrients daily excreted in faeces. The latter was calculated by multiplying DM, CP, and fat content of faeces by the total amount of faeces collected daily.

Growth performance was analysed using a mixed-effects model including the fixed effect of treatment, week of measurement, and their 2-way interaction, plus the random effect of animal using SAS (SAS Institute Inc., Cary, NC; 2002–2008, Release 9.2). Week of measurement entered the model as a repeated measure. The remaining data (milk composition, hindgut biopsies, and hindgut bacterial population) were analysed using a general linear model including the effect of treatment with the ANOVA statement of SAS (SAS Institute Inc., Cary, NC; 2002–2008, Release 9.2).

## Results and discussion

All data pertaining to 1 calf in the UHT treatment were excluded from the study because the calf suffered diarrhoea problems.

### *Milk composition*

Milk protein and fat composition did not differ among treatments (see Table S2 in Supplementary Files). The lack of differences in milk fat and protein contents was expected as all treatments were based on the same bulk milk, and heat treatment has no effect on total fat and protein contents. However, the content of furosine (an Amadori compound, which renders lysine unavailable for digestion) in milk was about 33-fold greater ($P < 0·001$) in UHT (33·8 ± 0·25 mg/l) than in RM and PAST milks (1·13 ± 0·25 mg/l). The amount of carboxylmehtyl-lysine was below the detection levels (0·1 mg/l) in RM and PAST milks, but was 0·17 ± 0·04 mg/l in UHT. Amadori products are formed during early stages of the Maillard reactions by the condensation of a lysine residue with a reducing sugar forming a Schiff's base (Van Boekel, 1998) and are not digested (Friedman, 1996). Thus, it can be inferred that heating milk for 6 s at 142 °C diminishes the amount of available lysine.

### *Intake, growth performance, digestion and nitrogen retention*

Total milk consumption was below the 154 l offered because during the first 6–7 d of study calves consumed less than the 6 l offered. Calves on UHT had greater ($P < 0·05$) milk left overs than calves on RM or PAST (Table 1); although during the last 3 d of study (when the N balance and digestibility measures took place) all calves, independently of treatment, consumed their entire milk portions (8 l/d). The reduction in milk consumption observed with UHT milk could be linked with a delay in abomasal emptying. Some authors have reported that heat treatment (>75 °C) increases buffering capacity of milk due to the solubilisation of colloidal calcium phosphate (Amador-Espejo et al. 2014) and this may compromise digestion and intake.

Straw intake was greatest in calves consuming RM (Table 1). This was mainly due to an interaction between treatment and time. Calves on RM consumed more straw (29·8 ± 2·51 g/d) during the third week of study than calves on PAST and UHT milks (13·8 ± 2·51 g/d), but the biological consequences of such small differences should be minimal as the nutritive value of straw is extremely poor.

There were no differences in initial body weight (BW); however, average daily gain (ADG) was lowest ($P < 0·01$) in UHT calves (Table 1). Interestingly, differences in ADG were larger than it could be anticipated based solely on differences in milk consumption. A 0·75 l/d difference in milk consumption (found between UHT and RM) is equivalent to about 94 g/d of solids from milk, which should support about 130 g/d of growth NRC (2001). The differences in

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**Table 2.** Faecal bacterial counts and health status of the hindgut mucosa (expressed relative to a randomly chosen sample from one calf receiving raw milk) of 3-week old calves as affected by the type of heat treatment applied to the milk consumed

| | Raw | Pasteurised | UHT | SE | *P*-value |
|---|---|---|---|---|---|
| Bacterial population | | | | | |
| *Lactobacillus* | 0·86[a] | 0·75[ab] | 0·68[b] | 0·05 | 0·04 |
| *Bifidobacterium* | 0·92 | 0·96 | 0·99 | 0·07 | 0·74 |
| *Faecalibacterium* | 1·09 | 1·30 | 0·97 | 0·15 | 0·33 |
| Gram positive | 0·92 | 0·92 | 0·86 | 0·04 | 0·43 |
| Gram negative | 0·97[ab] | 0·88[b] | 1·14[a] | 0·06 | 0·03 |
| Relative gene expression | | | | | |
| $TNF_{\alpha}$ | 3·75 | 5·98 | 4·55 | 1·44 | 0·55 |
| $TGF_{\beta}$ | 1·04 | 1·10 | 1·29 | 0·11 | 0·28 |
| IL-10 | 1·65 | 4·83 | 4·54 | 1·05 | 0·09 |
| IL-8 | 1·70[b] | 3·99[ab] | 7·20[a] | 1·37 | 0·05 |
| Occludin | 8·83 | 9·04 | 11·80 | 3·36 | 0·79 |
| β-defensin | 0·76 | 1·43 | 2·04 | 0·56 | 0·31 |
| TLR-4 | 1·40 | 2·06 | 2·81 | 0·70 | 0·39 |
| Claudin-4 | 1·55 | 1·64 | 4·36 | 41·2 | 0·06 |
| IL-1β | 0·90 | 0·71 | 0·69 | 0·29 | 0·85 |

[a,b]Values with uncommon superscripts differ at $P < 0·05$

ADG between RM and UHT were >200 g/d. Thus, other reasons beyond nutrient intake should be behind part of the differences in ADG observed between RM and UHT calves. Most likely, this reason was nutrient digestion or metabolic availability between treatments. The main change upon heating milk is denaturation of whey proteins and their interaction with casein micelles, and the ε-amino groups of some lysine residues may be blocked and become resistant to digestion by trypticase and other digestive enzymes (Rerat et al. 2002). In support of this fact, furosine content in milk herein was much greater in UHT than in RM. Wada & Lönnerdal (2014) reported an increase in protein digestibility *in vitro* when applying heat (UHT, steam infusion, and in-can sterilisation) to milk, which was mainly due to protein denaturation. But, interestingly, when heat-treated milks were offered to rat pups *in vivo* digestibility actually decreased. Authors attributed this decrease to reduced casein coagulation in the stomach and formation of disulphide bonds and protein aggregation leading to the formation of Amadori products.

Another factor potentially contributing to the worse growth performance of UHT calves, could be found in the greater urinary N excretion recorded with this treatment. Total tract digestibility of dry matter, fat, and protein did not differ among treatments (Table 1). However, urinary N excretion was greater ($P < 0·05$) in calves fed UHT than in those fed RM and PAST. Nitrogen retention (expressed as a percentage of total N intake) did not differ among treatments (Table 1). Lacroix et al. (2008) reported a greater anabolic use of milk N in plasma proteins and greater irreversible N losses in humans consuming UHT compared with pasteurised or micro-filtrated milk. They concluded that the increased N losses were probably due to increased lysine damage in UHT milk that would result in greater deamination level of dietary amino acids on one hand, and to an increased digestion kinetics of UHT milk leading to an increase in dietary N transfer into plasma protein and urea.

### *Bacterial populations and hindgut inflammation status*

There were no differences in the quantification of total Gram positive bacteria, *Bifidobacterium*, and *Faecalibacterium*, but *Lactobacillus* was lower in calves fed UHT or PAST milk than in those receiving RM (Table 2). *Lactobacilli* inhibit the growth of pathogenic microorganisms through mechanisms of competitive exclusion, stimulation of the host immune system, and production of specific antibacterial compounds, such as acetic and lactic acids (Elmadfa et al. 2010). By contrast, quantification of total Gram negative was greater in UHT than in PAST calves, with calves on RM showing intermediate values. The greater quantification of Gram negative bacteria in UHT calves compared with PAST could represent a threat to the intestinal barrier integrity due to lipopolysaccharide release and bacterial translocation (Maes et al. 2008).

Overall, there were no differences in the expression of the evaluated genes in the hindgut mucosa (Table 2), except for IL-8 that was expressed in greater amounts ($P = 0·05$) in UHT than in RM calves, with PAST having intermediate values. Claudin-4 was numerically greater in calves consuming UHT and IL-10, was numerically lowest in calves receiving RM. Interleukin-8 acts as a potent chemotactic molecule that attracts neutrophils and induces phagocytosis and enhances inflammation, and over-expression of IL-8 has been associated with bowel disease in humans (Mazzucchelli et al. 1994). On the other hand, IL-10 is an anti-inflammatory cytokine that plays a pivotal role in the function of regulatory T cells that control inflammatory responses in the intestine (Asseman et al. 1999). In other

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words, in the presence of an inflammatory response, IL-10 is needed to modulate the immune response. Thus, the tendency of RM calves to have a lower expression of IL-10 than PAST and UHT calves coupled with the increased expression of IL-8 in UHT than in RM calves could indicate that UHT calves may have had a greater inflammatory status in the hindgut. Lastly, the tendency for UHT calves to have a greater expression of claudin-4 compared with RM and PAST calves could be indicative of a poorer integrity of the hindgut mucosa in the former animals. Li et al. (2013) fed piglets four milk replacers identical in composition but differing in the heat treatment applied to their whey fractions and reported that lactose digestion and galactose absorption decreased as the severity of heat treatment increased. As a consequence, piglets fed the milk replacer with the most severe heat treatment (repeated pasteurisation) had increased intestinal damage as indicated by the greatest production of claudin-4.

## Conclusions

In conclusion, UHT treatment of milk may decrease its nutritional value because it may have deleterious effects on the inflammation status of the gut and alter its bacterial population, compromises growth, and increases N excretion in young calves.

## Supplementary material

The supplementary material for this article can be found at https://doi.org/10.1017/S0022029917000401

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