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Authors: Brendon D. Gill, Harvey E. Indyk, Marilyn Manley-Harris

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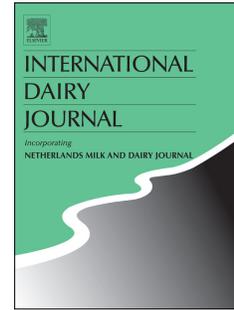
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1 **Determination of total potentially available nucleosides in bovine milk**

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5 Brendon D. Gill<sup>a,b,\*</sup>, Harvey E. Indyk<sup>a</sup>, Marilyn Manley-Harris<sup>b</sup>

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7 <sup>a</sup> *Fonterra Co-operative Group Ltd, P.O. Box 7, Waitoa 3341, New Zealand*

8 <sup>b</sup> *University of Waikato, Private Bag 3105, Hamilton 3240, New Zealand*

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11 \*Corresponding author: Tel.: +64 7 889 3989; fax: +64 7 887 1502

12 *E-mail address: [brendon.gill@fonterra.com](mailto:brendon.gill@fonterra.com) (B. D. Gill).*

13

14 **Abstract**

15

16 Bovine colostrum and milk samples were collected from two herds over  
17 the course of the first month post-partum, pooled for each herd by stage of  
18 lactation and total potentially available nucleosides were determined. Sample  
19 analysis consisted of parallel enzymatic treatments, phenylboronate clean-up,  
20 and liquid chromatography to quantify contributions of nucleosides,  
21 monomeric nucleotides, nucleotide adducts, and polymeric nucleotides to the  
22 available nucleosides pool. Bovine colostrum contained high levels of  
23 nucleosides and monomeric nucleotides, which rapidly decreased as lactation  
24 progressed into transitional milk. Mature milk was relatively consistent in  
25 nucleoside and monomeric nucleotide concentrations from approximately the  
26 tenth day post-partum. Differences in concentrations between summer-milk  
27 and winter-milk herds were largely attributable to variability in uridine and  
28 monomeric nucleotide concentrations.

29

## 30 1. Introduction

31

32 Nucleosides are low molecular weight compounds consisting of a purine  
33 or pyrimidine base (e.g., adenine, cytosine, guanine and uridine) attached via  
34 a  $\beta$ -glycosidic linkage to a ribose sugar (ribonucleosides). Nucleotides are *o*-  
35 phosphoric acid esters of nucleosides containing one to three phosphate  
36 groups on C-2, C-3 or most commonly C-5 of the ribose (ribonucleotides).

37 Nucleotides are compounds of critical importance to cellular function.  
38 They operate as precursors to nucleic acids, as mediators of chemical energy  
39 transfer and cell signalling, and as integral components of coenzymes in the  
40 metabolism of carbohydrates, lipids and proteins (Carver & Walker, 1995;  
41 Cosgrove, 1998).

42 Nucleotides can be synthesised *de novo* or recovered via salvage  
43 pathways and thus are not essential dietary nutrients. However, during  
44 periods of rapid growth or after injury, when the metabolic demand for  
45 nucleotides exceeds the combined capacity of *de novo* synthesis and the  
46 salvage pathway, dietary sources of nucleotides are considered to be  
47 conditionally essential for continued optimal metabolic function (Carver &  
48 Walker, 1995; Yu, 1998). Dietary nucleotides are ingested in the form of  
49 nucleoproteins, polymeric nucleotides (nucleic acids) and nucleotide adducts  
50 as well as free nucleotides. These are digested in the gastrointestinal tract by  
51 proteases, nucleases, phosphatases and nucleotidases, and are available for  
52 absorption predominantly as nucleosides (Quan, Barnes, & Uauy, 1990;  
53 Uauy, Quan, & Gil, 1994).

54 Dietary nucleotides have been shown to increase immune response in  
55 infants (Carver, Pimentel, Cox, & Barness, 1991; Pickering et al., 1998), to  
56 influence metabolism of long chain fatty acids and to enhance gastrointestinal  
57 tract repair after damage, when compared with nucleotide-unsupplemented  
58 diets (Carver & Walker, 1995; Gil, Corral, Martínez, & Molina, 1986). Dietary  
59 supplementation of infant formula with nucleotides has also been reported to  
60 beneficially modify the composition of intestinal microflora (Uauy et al., 1994),  
61 to elevate serum immunoglobulin concentrations and to reduce incidences of  
62 diarrhoea (Yau et al., 2003).

63 The expression of nucleosides and nucleotides in bovine milk is highest  
64 immediately after parturition with a general decreasing trend in concentration  
65 with advancing lactation, with levels stabilising by the third week of lactation  
66 (Gil & Sanchez-Medina, 1981; Gill & Indyk, 2007b; Schlimme, Martin, &  
67 Meisel, 2000; Sugawara, Sato, Nakano, Idota, & Nakajima, 1995). This  
68 pattern of high concentration in early colostrum followed by a rapid reduction  
69 as lactation progresses is analogous to changes of other bioactive  
70 components, such as immunoglobulins.

71 In general, the dominant strategy employed in analysis of free nucleosides  
72 and nucleotides in colostrum and milk has been protein removal by acid  
73 precipitation, followed by HPLC-UV analysis of the crude or fractionated  
74 extract (Ferreira, Mendes, Gomes, Faria, & Ferreira, 2001; Gill & Indyk,  
75 2007a, b; Sugawara et al., 1995).

76 Early clinical studies employed infant formulas containing nucleotides  
77 supplemented to levels based on estimates of the free nucleotide content of  
78 human milk (Aggett, Leach, Rueda, & MacLean, 2003). However, the  
79 measurement of free nucleotide levels does not account for nucleosides,

80 polymeric nucleotides or nucleotide adducts that are also nutritionally  
81 available to the infant. In order to determine the total potentially available  
82 nucleosides (TPAN), an analytical protocol to characterise the contributions of  
83 different molecular nucleoside sources to infant nutrition was developed  
84 (Leach, Baxter, Molitor, Ramstack, & Masor, 1995). The development of this  
85 protocol has been an important contribution to further understanding the  
86 distribution of nucleosides and nucleotides and their implications for infant  
87 nutrition. The analytical method uses a number of enzymatic treatments  
88 incorporating combinations of nuclease, pyrophosphatase and phosphatase  
89 enzymes into the sample preparation. In this manner, contributions from  
90 nucleoside precursors to TPAN in human milk have been estimated, and it  
91 was reported that the nutritionally relevant concentrations of nucleosides and  
92 nucleotides in human milk had been underestimated by approximately 50%  
93 when compared with free nucleotide concentrations only (Gerichhausen,  
94 Aeschlimann, Baumann, Inäbnit, & Infanger, 2000; Leach et al., 1995;  
95 Tressler et al., 2003).

96 Bovine milk is almost exclusively used in the manufacture of infant formula  
97 intended to substitute for human breast milk, and since the levels of TPAN in  
98 bovine milk have not been previously reported, the purpose of the current  
99 study was to evaluate bovine milk TPAN levels and variation over the first  
100 month of lactation.

## 101 2. Materials and methods

102

### 103 2.1. Apparatus

104

105 The high performance liquid chromatography (HPLC) system consisted of  
106 an SCL-10Avp system controller, LC-10ADvp pump, FCV-10ALvp low  
107 pressure gradient unit, SIL-10AF sample injector unit equipped with a 50  $\mu$ L  
108 injection loop, DGU-14A degasser unit, CTO-10ASvp column oven and SPD-  
109 M10Avp photodiode array detector (Shimadzu, Kyoto, Japan). Instrument  
110 control and data processing were implemented using Shimadzu Class-VP  
111 version 6.12.

112 The column selected was a Prodigy C<sub>18</sub> column, 5  $\mu$ m, 4.6  $\times$  150 mm  
113 (Phenomenex, Torrance, CA, USA). Prior to use, mobile phases were filtered  
114 and degassed using a filtration apparatus with 0.45  $\mu$ m nylon filter  
115 membranes (AllTech, Deerfield, IL, USA). Solid phase extraction of  
116 nucleosides was performed using Affi-gel 601 (Bio-Rad, Hercules, CA, USA).

117

### 118 2.2. Reagents

119

120 Adenosine, cytidine, guanosine, uridine, 5-methylcytidine, uridine  
121 5'-diphosphoglucose, RNA, cytidine 5'-diphosphocholine,  $\beta$ -nicotinamide  
122 adenine dinucleotide, adenosine 5'-monophosphate (AMP), cytidine  
123 5'-monophosphate (CMP), guanosine 5'-monophosphate (GMP), uridine  
124 5'-monophosphate (UMP) nuclease P1, pyrophosphatase, and alkaline  
125 phosphatase were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

126 Potassium dihydrogen phosphate, orthophosphoric acid, hydrochloric acid,  
127 sodium hydroxide and potassium hydroxide were supplied by Merck  
128 (Darmstadt, Germany). Water was purified with resistivity  $\geq 18 \text{ M}\Omega$  using an  
129 E-pure water system (Barnstead, IA, USA).

130

### 131 2.3. *Sample collection*

132

133 Milk and colostrum samples were collected from seven cows from each of  
134 two Jersey herds from two separate farms in the eastern Waikato region of  
135 New Zealand. Samples from a winter-milk herd were collected over a 1  
136 month period in late March 2008 and samples from a summer-milk herd were  
137 collected over a 1 month period in early August 2009. Cows selected for  
138 inclusion in this study were in general good health, in their second or  
139 subsequent calving and had experienced normal calvings without  
140 complications. With the exception of the 6 h sample, sample collection was  
141 performed between 6:00 and 10:00 am, which coincided with regular morning  
142 milking times.

143 From each cow, approximately 80 mL of sample was collected in a  
144 120 mL disposable container. These samples were collected at various time  
145 intervals throughout the first month of lactation, with a frequency that reduced  
146 as the month progressed.

147 Collected samples were refrigerated at 4 °C, picked up from the farm as  
148 soon as practicable (within 6 h), taken to the laboratory and immediately  
149 prepared for storage. NaOH (1 M, 20 mL) was added to a 10 mL sample  
150 aliquot and mixed, and the sample was then left to stand for 30 min,

151 neutralised to  $\text{pH} = 7.35 \pm 0.05$  with HCl and made to 50 mL volume before  
152 freezing at  $< -15$  °C.

153

#### 154 2.4. *Sample analysis*

155

156 Samples from the seven cows at each time period post-partum were  
157 pooled for analysis, and enzymatic hydrolysis and boronate affinity extraction  
158 were performed as described by Leach et al. (1995). Each pooled sample  
159 was tested in duplicate with the mean and standard deviation calculated.

160 Samples were enzymatically hydrolysed using nucleotide  
161 pyrophosphatase, nuclease P1 and bacterial alkaline phosphatase (Sigma  
162 Chemical Co., St. Louis, MO, USA). Each pooled sample was split into four  
163 5 mL sub-samples, to each of which internal standard (10  $\mu\text{g}$ , 5-methylcytidine)  
164 was added, and each sub-sample was subjected to a different enzymatic  
165 treatment. The first treatment had no added enzymes and innate nucleosides  
166 only were therefore measured. The second treatment involved phosphatase  
167 ( $\text{pH} = 8.5$ , 3 h), which dephosphorylated monomeric nucleotides to  
168 nucleosides. The third treatment incorporated nuclease ( $\text{pH} = 5.1$ , 16 h) and  
169 phosphatase ( $\text{pH} = 8.5$ , 3 h), which hydrolysed polymeric nucleotides to  
170 monomeric nucleotides, which were subsequently dephosphorylated to  
171 nucleosides. The fourth treatment consisted of nuclease ( $\text{pH} = 5.1$ , 16 h),  
172 pyrophosphatase and phosphatase ( $\text{pH} = 8.5$ , 3 h), which converted all  
173 nucleoside precursors (polymeric and monomeric nucleotides, and nucleotide  
174 adducts) to free nucleosides.

175 Clean-up of enzymatic extracts was achieved by solid phase extraction  
176 using a phenylboronate affinity gel as described by Leach et al. (1995),

177 whereby nucleosides were covalently bonded to the gel at high pH, and  
178 interferences removed with two washings of high pH buffer. The nucleosides  
179 were eluted from the affinity gel at low pH by the addition of phosphoric acid  
180 (0.25 M), and filtered ready for analysis (Liu & Scouten, 2000).

181

## 182 2.5. *Chromatographic analysis*

183

184 The initial chromatographic protocol was a modification of a reversed-  
185 phase system described by Gill and Indyk (2007b), using phosphate buffer  
186 and a methanol gradient. As optimum separation of nucleosides was  
187 achieved at pH = 4.8, phosphate was replaced with acetate (pKa = 4.75),  
188 thereby offering greater buffer capacity at the desired pH.

189 An organic solvent component is required in the mobile phase to facilitate  
190 elution of nucleosides from the C<sub>18</sub> column. However, to obtain sufficient  
191 resolution between peaks, a gradient elution procedure was necessary. A  
192 number of gradient procedures were evaluated to determine an optimum  
193 protocol that had a relatively short run-time coupled with sufficient resolution  
194 between peaks. An optimum separation of nucleosides was achieved at a  
195 flow rate of 0.7 mL min<sup>-1</sup> with gradients formed by low pressure mixing of two  
196 mobile phases, A (0.05 M sodium acetate, pH = 4.8) and B (100% methanol)  
197 (0–3 min, 95:5, v/v, A:B; 7–22 min 75:25, v/v, A:B; 23–30 min 95:5, v/v, A:B).

198 The photodiode array detector acquired spectral data between 210 and  
199 300 nm. Peak identification was by co-chromatography and similarity of the  
200 chromatographic peak spectrum to authentic standards, as estimated by a  
201 similarity index of > 0.99. Chromatograms were integrated at a wavelength of

202 260 nm and results were determined by an internal standard technique using  
203 5-methylcytidine.

204 The contributions of the different forms (nucleosides, nucleotide adducts,  
205 monomeric and polymeric nucleotides) to TPAN were calculated in the  
206 manner described by Leach et al. (1995) using Excel spreadsheet software  
207 (Microsoft, Redmond, WA, USA).

208

## 209 2.6. Recovery

210

211 A spiked recovery study was performed on free nucleosides and was  
212 assessed through the affinity gel sample clean-up. A stored pooled milk  
213 sample was spiked with a single mixed standard containing cytidine,  
214 guanosine, uridine, adenosine and 5-methylcytidine ( $95.0\text{--}135.0\ \mu\text{g mL}^{-1}$ ).  
215 Recovery was assessed by comparison of peak areas for the spiked and  
216 unspiked samples, relative to those of the mixed standard.

217 Recovery of nucleosides from the enzymatic digestion was estimated  
218 following the protocol described by Leach et al. (1995). A solution  
219 (TPAN-fortified) containing ribonucleosides, 5'-mononucleotides, nucleotide  
220 adducts and RNA was prepared for a spiked recovery study. A solution  
221 (TPAN-digest) was made from an aliquot (5 mL) of the TPAN-fortified solution  
222 that was hydrolysed for 20 h with KOH ( $0.2\ \text{mol L}^{-1}$ , 50 mL) to convert  
223 polymeric RNA to monomeric nucleotides. The pH of the solution was  
224 adjusted to 9.0 with HCl and then incubated with alkaline phosphatase and  
225 nucleotide pyrophosphatase to convert adducts and monomeric nucleotides to  
226 nucleosides. The concentration of nucleosides in the TPAN-digest solution

227 was determined by HPLC and was used to calculate the TPAN content in the  
228 TPAN-fortified solution.

229 A stored pooled milk sample was then spiked (in triplicate) with an aliquot  
230 of the TPAN-fortified solution and, along with unspiked sample replicates, was  
231 analysed and TPAN concentrations determined. Recovery was assessed by  
232 comparison of the difference in results for the spiked and unspiked samples,  
233 divided by the TPAN concentration of the TPAN-fortified solution.

234

### 235 2.7. Statistical analysis

236

237 The experimental data were analysed by one-way analysis of variance  
238 (ANOVA) of the response of season (winter-milk, summer-milk) with covariate  
239 time (0, 0.25, 1, 2, 3, 5, 10, 20, 30 days post-partum). All results ( $X$ ) were  
240 transformed  $\log_{10}(1 + X)$ , so that the postulated model was an exponential  
241 decrease in levels with time, with the initial levels and the rates of decrease  
242 dependent upon season. The “exponential decay” model was found to  
243 provide a better fit than a linear or quadratic model in time. For hypothesis  
244 testing, significance was evaluated at the  $P < 0.05$  level. Statistical analyses  
245 were performed using Minitab version 15.1 (State College, PA, USA).

## 246 3. Results and Discussion

247

### 248 3.1. Recovery

249

250 The recoveries of nucleosides (recovery  $\pm$  standard deviation) through the  
251 affinity gel clean-up were as follows: cytidine ( $93.4 \pm 1.1\%$ ), uridine  
252 ( $92.3 \pm 5.1\%$ ), guanosine ( $88.3 \pm 4.9\%$ ), adenosine ( $95.2 \pm 4.2\%$ ), and  
253 5-methylcytidine ( $92.6 \pm 2.3\%$ ). Recoveries measured through the enzymatic  
254 digestion and subsequent affinity gel clean-up were: cytidine ( $95.5 \pm 2.8\%$ ),  
255 uridine ( $101.7 \pm 3.7\%$ ), guanosine ( $89.2 \pm 2.4\%$ ) and adenosine ( $94.7 \pm 3.0\%$ ).  
256 These recovery values were acceptable for the quantitative analysis of  
257 nucleosides at concentrations typical of bovine milk samples (AOAC, 2002).

258

### 259 3.2. Chromatography

260

261 Chromatographic performance evaluated as resolution, peak tailing,  
262 retention factor, and peak area repeatability, was deemed acceptable by  
263 replicate analyses ( $n = 6$ ) of a mixed nucleotide standard (Fig. 1A). The  
264 specificity of the phenylboronate sample clean up provides analytical  
265 chromatography relatively free of interferences (Fig. 1B).

266

### 267 3.3. Total potentially available nucleosides in bovine milk

268

269 The TPAN concentrations and contribution of each nucleobase and form  
270 obtained in this study of winter-milk and summer-milk lactation series are  
271 summarised in Tables 1 and 2 and illustrated graphically in Figs. 2 and 3. For

272 each parameter (each base within each form), comparisons of the initial levels  
273 and rates of decrease were made between seasons and whether each  
274 seasonal slope differed from zero (Table 3).

275

### 276 3.3.1. Nucleoside contribution to TPAN

277 Uridine was the most prevalent nucleoside, at levels of  $\sim 50 \mu\text{mol dL}^{-1}$  in  
278 colostrum, but these levels were not sustained beyond the third day post-  
279 partum and rapidly decreased to levels similar to those of cytidine and  
280 guanosine, at  $1\text{--}3 \mu\text{mol dL}^{-1}$ . Adenosine was present at much lower levels  
281 but these low levels were maintained throughout the lactation period for both  
282 seasons milk. The nucleoside levels measured in this study were consistent  
283 with those reported previously (Gill & Indyk, 2007b). Although nucleosides  
284 were present at higher concentrations in bovine colostrum than in mature  
285 bovine milk, they rapidly decreased to levels similar to that in mature human  
286 milk, as reported by Leach et al. (1995).

287

### 288 3.3.2. Monomeric nucleotide contribution to TPAN

289 Levels of nucleotides measured in this study were generally higher than  
290 those reported previously (Gill & Indyk, 2007b); however, there was likely to  
291 have been a significant contribution from multiple phosphorylated forms  
292 (cyclic-, mono-, di- and tri-phosphorylated nucleotides), which the TPAN  
293 analytical method aggregates as a single value. Differences in colostrum  
294 monomeric nucleotide levels between the herds were evident, with the winter-  
295 milk herd initially containing 5–10 times the levels of the summer-milk herd.

296 However, by the fifth day, nucleotide levels decreased to approximately  
297  $15 \mu\text{mol dL}^{-1}$  in both herds, somewhat lower than those reported in human  
298 milk (Leach et al., 1995). The high initial uridine nucleotides levels and  
299 subsequent rapid decrease in concentration seen in winter-milk was absent in  
300 summer-milk which maintained constant levels throughout lactation. Cytidine  
301 and adenosine nucleotides are stable throughout lactation for both seasons.  
302 The most abundant nucleotides in bovine colostrum were based on uridine;  
303 however, as colostrum transitioned into mature milk, cytidine nucleotides  
304 became the dominant form.

305 Uridine nucleotides are critical components in the biosynthesis of lactose.  
306 As lactose is a major osmotic component of milk, there is a correlation  
307 between the amount of lactose and the volume of milk produced (Arthur, Kent,  
308 & Hartmann, 1991; Linzell & Peaker, 1971). It has been suggested that high  
309 levels of uridine and UMP are present in milk, as breakdown products of  
310 uridine diphosphate (UDP) and uridine triphosphate (UTP), due to their  
311 function in the synthesis of lactose (Mateo, Peters, & Stein, 2004; Schlimme  
312 et al., 2000). It has been proposed that support for this hypothesis is seen by  
313 the correlation of decreasing total milk solids and 5'-UMP concentrations in  
314 sow's milk as lactation progresses (Mateo et al., 2004). However, as  
315 colostrum contains higher total milk solids and lower lactose levels (on a dry  
316 weight basis) than mature milk (Heng, 1999), a reduced proportion of uridine  
317 nucleotides than in mature milk might be expected based on this proposal.  
318 Alternative reasons must therefore be sought to account for the higher relative  
319 proportions of uridine nucleotides in colostrum. It has also been suggested  
320 that uridine accounts for many of the immunological properties of nucleotides  
321 in colostrum (Kulkarni, Fanslow, Rudolph, & Van Buren, 1986; Leach et al.,

1995; Van Buren, Kulkarni, Fanslow, & Rudolph, 1985) and, more recently, Mashiko et al. (2009) demonstrated that dietary UMP affected the immune response of newborn calves.

325

### 3.3.3. Nucleotide adduct contribution to TPAN

The results for uridine adducts in the present study ranged from not detected to  $23.7 \mu\text{mol dL}^{-1}$  in the winter-milk herd and from not detected to  $6.8 \mu\text{mol dL}^{-1}$  in the summer-milk herd, with a rapid reduction in concentration after the third day post-partum. Guanosine adducts measured ranged from not detected to  $3.9 \mu\text{mol dL}^{-1}$  in the winter-milk herd and from not detected to  $1.2 \mu\text{mol dL}^{-1}$  in the summer-milk herd. Similar levels of adenosine adducts were found, presumably derived from flavin adenine dinucleotide and nicotinamide adenine dinucleotide (Fox & McSweeney, 1998; Kanno, Shirahuji, & Hoshi, 1991). Utilising enzymatic techniques, Gil and Sánchez-Medina (1981) measured UDP hexosamine, UDP hexose and UDP galactose concentrations in bovine colostrum and milk, which ranged from not detected to  $\sim 104 \mu\text{mol dL}^{-1}$ . Levels were highest at 27 and 78 h and much lower or absent in subsequent stages of lactation. Guanosine diphosphate fucose was also reported at 27 and 78 h, at levels of  $6.7$  and  $4.1 \mu\text{mol dL}^{-1}$ , respectively.

341

### 3.3.4. Polymeric nucleotide contribution to TPAN

The concentration of polymeric nucleotides in bovine colostrum was similar to that in human colostrum and milk, however, with advancing lactation, the levels in bovine milk decreased below those in human milk. Both cytidine

346 and uridine contributions to polymeric nucleotides are steady throughout  
347 lactation for summer-milk, whereas the higher initial levels of polymeric uridine  
348 shows distinct decrease in concentration as lactation progresses in winter-milk.  
349

### 350 3.3.5. Nucleobase contribution to TPAN

351 Differences in the contributions of each nucleobase from the various  
352 nucleoside and nucleotide forms were found. The pyrimidines differed  
353 markedly from each other through lactation. Whereas the quantities of  
354 cytidine and cytidine nucleotides were relatively constant throughout, uridine  
355 and uridine nucleotides levels varied considerably. Cytidine concentrations  
356 were similar to those in human milk reported by Leach et al. (1995), whereas  
357 uridine was present at considerably higher levels in bovine colostrum and in  
358 lower amounts in mature bovine milk.

359 The concentrations of the purines also differed with adenosine levels  
360 throughout the first month of lactation for milk from both herds, whereas  
361 guanosine showed a significant decrease in levels for both herds. The  
362 quantities of both guanosine and adenosine, and their respective nucleotides  
363 were slightly higher in bovine colostrum than in human colostrum and milk, but  
364 concentrations were lower as colostrum transitioned to mature milk. In bovine  
365 milk, purine nucleosides and nucleotides made a relatively small contribution  
366 to TPAN (6–20%), whereas human milk purine nucleosides and nucleotides  
367 consistently represent a greater proportion of TPAN (> 30%).

368

369 3.3.6. *Total potentially available nucleosides*

370

371 In general, the absolute concentrations indicated a distinct difference  
372 between the two herds, although the general trends were the same. Winter  
373 had higher initial levels of TPAN but the rate of decrease was greater, such  
374 that the seasonal differences in TPAN concentration found in colostrum were  
375 largely absent in mature milk.

376 TPAN levels in winter-milk colostrum were attributable largely to  
377 significantly higher amounts of uridine nucleotides compared with summer-  
378 milk colostrum; however, by the tenth day, milk from both herds showed  
379 similar TPAN levels. The TPAN levels in bovine colostrum were higher than  
380 those in both human colostrum and milk, however, after transition to mature  
381 milk, the TPAN levels were lower than those reported in human milk (Leach et  
382 al., 1995).

383 It has been reported that nucleotides in human milk exhibit a circadian  
384 rhythmicity (Sánchez et al., 2009). Anomalous results for uridine and uridine  
385 nucleotides were found in bovine colostrum samples collected from both  
386 herds at 6 h post-partum, and such diurnal variation may suggest a plausible  
387 rationale given that this sample was uniquely collected in the afternoon.

388 The levels and distribution of TPAN in mature bovine milk are important in  
389 the manufacture of infant formulas, particularly when formulating to TPAN  
390 regulatory limits. If all endogenous forms of nucleosides and nucleotides that  
391 contribute to TPAN are not accounted for prior to nucleotide supplementation,  
392 possible over-fortification could occur during the manufacture of bovine milk-  
393 based infant formula.

394

395 3.4. *Herd conditions*

396

397 Although the feeding practices were similar on both farms, it is possible  
398 that seasonal or pasture differences could have had a significant effect on the  
399 nucleoside precursors expressed in the milk of each herd. Prior to calving,  
400 the cows' diet was extensive grass grazing supplemented with maize silage  
401 and palm kernel, and after calving, intake of grass and palm kernel increased  
402 with inclusion of whey permeate. One uncontrolled variable that may have  
403 had a profound influence is the climate. Calving for the winter-milk herd  
404 began in the early autumn of 2008, which followed a summer characterised by  
405 a La Niña weather pattern that contributed to record high temperatures and a  
406 drought with severe soil moisture deficits in the Waikato region of New  
407 Zealand. The summer-milk herd began calving in late winter 2009, which had  
408 the warmest August on record, although rainfall was normal (National Institute  
409 of Water and Atmospheric Research [NIWA], 2010). In addition to obvious  
410 climatic factors, other factors could have affected TPAN levels in both herds,  
411 such as the conditions under which the cows were raised and fed, tolerance to  
412 stress, sunlight exposure and other environmental factors. Further study  
413 controlling each of these factors would be required to identify those factors  
414 that influence nucleoside and nucleotide expression in milk. Limitations of the  
415 current study could be expanded upon in future experiments that consider the  
416 effects of breed, location and diet on TPAN expression in milk.

**417 4. Conclusions**

418

419 Nucleosides and monomeric nucleotides were the dominant forms of  
420 TPAN in bovine milk and colostrum, whereas nucleotide adducts and  
421 polymeric nucleotides contributed relatively little. Uridine and uridine  
422 nucleotides were the major contributor to TPAN in early colostrum, and  
423 cytidine and cytidine nucleotides dominated later in lactation. Differences in  
424 TPAN concentrations between summer-milk and winter-milk herds were  
425 largely attributable to variability in uridine and nucleotide concentrations. As  
426 lactation progressed, TPAN concentration decreased, as did each of the  
427 contributing forms.

428 With the increasing trend towards nucleotide supplementation of bovine  
429 milk-based infant formulas, and the need for compliance with TPAN regulatory  
430 limits, the data presented in this study provide a greater understanding of the  
431 contributions of endogenous nucleosides and nucleotides in bovine milk. In  
432 addition, colostrum is increasingly being used as a dietary supplement and the  
433 high level of TPAN present may be nutritionally significant.

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435

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Table 1.

Nucleosides and nucleotides in bovine milk from a winter-milk herd ( $\mu\text{mol dL}^{-1}$ )<sup>a</sup>.

Day <sup>b</sup>	Form <sup>c</sup>	Cytidine	Uridine	Guanosine	Adenosine	Total
0	Nucleoside	5.4 ± 0.1	57.9 ± 1.6	0.3 ± 0.0	nd	63.6 ± 1.5
	Monomeric NT	6.1 ± 0.3	143.7 ± 8.5	2.8 ± 0.0	2.9 ± 0.2	155.5 ± 8.7
	NT Adduct	0.9 ± 0.2	23.7 ± 9.0	3.9 ± 0.8	2.4 ± 0.0	30.9 ± 9.6
	Polymeric NT	0.6 ± 0.0	5.4 ± 7.2	1.4 ± 0.2	1.4 ± 0.1	8.7 ± 7.4
	Total Base	13.0 ± 0.5	230.7 ± 6.1	8.5 ± 0.9	6.6 ± 0.2	258.7 ± 6.8
+0.25	Nucleoside	4.0 ± 0.2	39.8 ± 0.2	0.2 ± 0.0	nd	44.0 ± 0.4
	Monomeric NT	1.3 ± 0.4	26.9 ± 4.7	1.0 ± 0.0	1.4 ± 0.0	30.6 ± 5.0
	NT Adduct	0.9 ± 0.2	3.2 ± 0.9	1.1 ± 0.2	0.5 ± 0.1	5.8 ± 0.6
	Polymeric NT	0.1 ± 0.0	3.9 ± 1.1	1.1 ± 0.1	0.9 ± 0.0	6.0 ± 0.9
	Total Base	6.3 ± 0.0	73.8 ± 4.3	3.5 ± 0.1	2.8 ± 0.1	86.4 ± 4.3
+1	Nucleoside	3.5 ± 0.1	49.8 ± 0.8	0.5 ± 0.0	nd	53.9 ± 0.7
	Monomeric NT	13.1 ± 0.3	77.5 ± 2.8	4.0 ± 0.2	3.0 ± 0.2	97.5 ± 3.2
	NT Adduct	0.4 ± 0.2	11.9 ± 6.8	2.4 ± 0.2	2.0 ± 0.6	16.5 ± 7.6
	Polymeric NT	0.5 ± 0.5	3.0 ± 3.8	1.3 ± 0.2	1.5 ± 0.5	6.4 ± 3.6
	Total Base	17.5 ± 0.9	142.2 ± 6.6	8.1 ± 0.0	6.5 ± 0.3	174.4 ± 7.9
+2	Nucleoside	2.5 ± 0.3	60.4 ± 0.4	0.8 ± 0.0	0.6 ± 0.1	64.2 ± 0.8
	Monomeric NT	16.9 ± 0.6	30.4 ± 3.4	2.0 ± 0.1	2.6 ± 0.0	51.6 ± 4.2
	NT Adduct	0.3 ± 0.2	6.7 ± 1.4	2.4 ± 0.2	2.6 ± 0.3	12.0 ± 1.3
	Polymeric NT	1.0 ± 0.1	2.7 ± 1.3	1.0 ± 0.1	1.2 ± 0.1	6.0 ± 1.3
	Total Base	20.7 ± 0.0	99.8 ± 3.2	6.2 ± 0.1	7.1 ± 0.3	133.8 ± 3.4
+3	Nucleoside	2.0 ± 0.2	42.7 ± 2.0	0.5 ± 0.1	0.6 ± 0.1	45.9 ± 2.4
	Monomeric NT	16.2 ± 0.4	22.2 ± 3.4	1.5 ± 0.2	3.6 ± 0.9	43.5 ± 4.9
	NT Adduct	0.4 ± 0.5	5.9 ± 0.3	2.2 ± 0.2	2.3 ± 0.1	10.7 ± 0.9
	Polymeric NT	0.3 ± 0.1	1.0 ± 0.3	0.6 ± 0.1	0.5 ± 0.6	2.5 ± 0.4
	TPAN	19.0 ± 0.2	71.8 ± 1.5	4.8 ± 0.2	7.0 ± 0.2	102.6 ± 1.2
+5	Nucleoside	1.5 ± 0.3	21.5 ± 0.8	nd	0.2 ± 0.0	23.3 ± 0.5
	Monomeric NT	12.1 ± 0.3	1.4 ± 0.1	0.6 ± 0.0	3.3 ± 0.1	17.4 ± 0.3
	NT Adduct	0.1 ± 0.0	0.8 ± 0.0	0.6 ± 0.2	0.6 ± 0.2	2.2 ± 0.4
	Polymeric NT	0.5 ± 0.1	0.4 ± 0.4	0.8 ± 0.1	0.7 ± 0.1	2.4 ± 0.5
	Total Base	14.1 ± 0.3	24.2 ± 1.0	2.1 ± 0.1	4.8 ± 0.3	45.2 ± 1.7
+10	Nucleoside	0.8 ± 0.2	3.2 ± 0.2	nd	0.1 ± 0.0	4.1 ± 0.0
	Monomeric NT	6.9 ± 0.3	0.4 ± 0.0	0.2 ± 0.0	2.4 ± 0.1	9.9 ± 0.4
	NT Adduct	0.1 ± 0.1	0.2 ± 0.2	0.1 ± 0.0	0.2 ± 0.1	0.6 ± 0.4
	Polymeric NT	0.3 ± 0.4	0.1 ± 0.1	0.4 ± 0.1	0.2 ± 0.0	1.0 ± 0.6
	Total Base	8.0 ± 0.1	3.9 ± 0.2	0.7 ± 0.1	3.0 ± 0.2	15.6 ± 0.2
+20	Nucleoside	0.7 ± 0.2	1.3 ± 0.1	nd	0.1 ± 0.0	2.1 ± 0.3
	Monomeric NT	3.9 ± 0.0	0.1 ± 0.1	nd	0.8 ± 0.1	4.8 ± 0.2
	NT Adduct	0.1 ± 0.0	0.2 ± 0.1	0.1 ± 0.1	0.1 ± 0.0	0.4 ± 0.2
	Polymeric NT	0.2 ± 0.1	0.1 ± 0.0	0.3 ± 0.0	0.2 ± 0.1	0.7 ± 0.2
	Total Base	4.8 ± 0.2	1.6 ± 0.1	0.4 ± 0.0	1.3 ± 0.2	8.0 ± 0.1
+30	Nucleoside	0.6 ± 0.1	0.8 ± 0.1	nd	0.1 ± 0.0	1.5 ± 0.0
	Monomeric NT	2.5 ± 0.0	0.1 ± 0.0	nd	0.3 ± 0.1	3.0 ± 0.1
	NT Adduct	nd	nd	nd	0.1 ± 0.0	0.2 ± 0.1
	Polymeric NT	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.7 ± 0.1
	Total Base	3.4 ± 0.0	1.1 ± 0.0	0.3 ± 0.0	0.6 ± 0.1	5.3 ± 0.1

<sup>a</sup> Values are given as the mean ± standard deviation of duplicate analyses; nd, not detected.<sup>b</sup> Day post-partum ± 2 h.<sup>c</sup> NT, nucleotide.

Table 2.

Nucleosides and nucleotides in bovine milk from a summer-milk herd ( $\mu\text{mol dL}^{-1}$ )<sup>a</sup>.

Day <sup>b</sup>	Form <sup>c</sup>	Cytidine	Uridine	Guanosine	Adenosine	Total
0	Nucleoside	2.6 ± 0.2	50.6 ± 5.8	2.2 ± 0.3	nd	55.4 ± 5.8
	Monomeric NT	1.5 ± 0.1	1.2 ± 0.0	0.2 ± 0.0	nd	2.8 ± 0.2
	NT Adduct	0.1 ± 0.1	0.5 ± 0.1	0.3 ± 0.0	0.2 ± 0.0	1.1 ± 0.2
	Polymeric NT	0.4 ± 0.0	0.3 ± 0.3	1.1 ± 0.0	0.9 ± 0.0	2.7 ± 0.3
	Total Base	4.7 ± 0.2	52.5 ± 6.1	3.7 ± 0.3	1.2 ± 0.0	62.1 ± 6.2
+0.25	Nucleoside	3.6 ± 0.1	28.0 ± 0.4	1.8 ± 0.0	nd	33.4 ± 0.5
	Monomeric NT	0.5 ± 0.3	0.4 ± 0.1	0.1 ± 0.0	nd	1.0 ± 0.5
	NT Adduct	0.2 ± 0.0	0.9 ± 0.2	0.1 ± 0.0	0.1 ± 0.0	1.4 ± 0.1
	Polymeric NT	0.3 ± 0.0	1.7 ± 0.3	0.8 ± 0.1	0.8 ± 0.0	3.6 ± 0.2
	Total Base	4.7 ± 0.2	31.0 ± 0.8	2.9 ± 0.2	0.9 ± 0.1	39.4 ± 0.3
+1	Nucleoside	5.4 ± 0.4	40.9 ± 1.2	2.1 ± 0.2	nd	48.5 ± 1.0
	Monomeric NT	7.3 ± 0.1	4.3 ± 0.3	0.3 ± 0.0	nd	11.9 ± 0.2
	NT Adduct	1.6 ± 0.3	6.8 ± 0.9	1.2 ± 0.1	0.3 ± 0.1	10.0 ± 1.2
	Polymeric NT	0.6 ± 0.1	1.1 ± 0.4	1.0 ± 0.1	0.7 ± 0.3	3.4 ± 0.0
	Total Base	15.0 ± 0.4	53.1 ± 0.4	4.7 ± 0.1	1.0 ± 0.3	73.8 ± 0.4
+2	Nucleoside	3.7 ± 0.4	39.2 ± 0.1	2.7 ± 0.4	nd	45.6 ± 0.9
	Monomeric NT	10.4 ± 0.8	0.4 ± 0.1	0.2 ± 0.0	0.9 ± 0.1	11.8 ± 1.0
	NT Adduct	nd	1.7 ± 0.4	0.9 ± 0.0	0.4 ± 0.0	2.9 ± 0.4
	Polymeric NT	0.5 ± 0.0	1.0 ± 0.0	0.4 ± 0.1	0.3 ± 0.0	2.3 ± 0.0
	Total Base	14.5 ± 0.4	42.3 ± 0.4	4.2 ± 0.4	1.5 ± 0.2	62.6 ± 0.2
+3	Nucleoside	6.7 ± 0.2	21.5 ± 1.6	1.2 ± 0.1	nd	29.4 ± 1.3
	Monomeric NT	5.8 ± 0.8	3.6 ± 0.8	0.3 ± 0.0	2.1 ± 0.4	11.9 ± 1.2
	NT Adduct	0.1 ± 0.0	0.5 ± 0.0	0.4 ± 0.1	0.4 ± 0.0	1.5 ± 0.1
	Polymeric NT	0.5 ± 0.1	1.4 ± 0.5	0.4 ± 0.0	0.3 ± 0.0	2.7 ± 0.6
	Total Base	13.2 ± 0.7	27.0 ± 3.0	2.3 ± 0.1	2.9 ± 0.4	45.3 ± 3.4
+5	Nucleoside	1.0 ± 0.1	9.2 ± 0.1	0.2 ± 0.3	nd	10.4 ± 0.3
	Monomeric NT	8.0 ± 0.2	0.4 ± 0.0	0.2 ± 0.0	2.0 ± 0.1	10.7 ± 0.1
	NT Adduct	0.3 ± 0.2	0.4 ± 0.0	0.1 ± 0.0	0.3 ± 0.0	1.0 ± 0.3
	Polymeric NT	0.8 ± 0.2	0.5 ± 0.1	0.3 ± 0.0	0.2 ± 0.1	1.9 ± 0.2
	Total Base	10.2 ± 0.1	10.5 ± 0.3	0.8 ± 0.3	2.4 ± 0.0	24.0 ± 0.1
+10	Nucleoside	0.6 ± 0.1	3.0 ± 0.0	nd	nd	3.6 ± 0.0
	Monomeric NT	4.1 ± 0.2	0.1 ± 0.0	nd	1.2 ± 0.1	5.3 ± 0.0
	NT Adduct	0.2 ± 0.1	nd	0.1 ± 0.0	0.2 ± 0.1	0.5 ± 0.2
	Polymeric NT	0.2 ± 0.1	0.2 ± 0.0	0.4 ± 0.0	0.1 ± 0.0	0.9 ± 0.1
	Total Base	5.0 ± 0.4	3.4 ± 0.0	0.4 ± 0.0	1.5 ± 0.1	10.3 ± 0.4
+20	Nucleoside	0.7 ± 0.0	1.5 ± 0.5	nd	nd	2.1 ± 0.5
	Monomeric NT	3.0 ± 0.2	0.1 ± 0.0	nd	0.4 ± 0.0	3.4 ± 0.1
	NT Adduct	0.1 ± 0.0	0.1 ± 0.0	nd	0.1 ± 0.0	0.2 ± 0.0
	Polymeric NT	nd	0.1 ± 0.0	nd	0.1 ± 0.0	0.1 ± 0.0
	Total Base	3.8 ± 0.2	1.6 ± 0.5	nd	0.5 ± 0.1	5.9 ± 0.4
+30	Nucleoside	0.6 ± 0.0	1.3 ± 0.0	nd	nd	1.9 ± 0.0
	Monomeric NT	1.6 ± 0.2	nd	nd	nd	1.6 ± 0.2
	NT Adduct	0.1 ± 0.0	0.1 ± 0.0	nd	0.3 ± 0.0	0.5 ± 0.0
	Polymeric NT	nd	nd	nd	nd	0.1 ± 0.0
	Total Base	2.3 ± 0.2	1.4 ± 0.0	nd	0.3 ± 0.0	4.0 ± 0.2

<sup>a</sup> Values are given as the mean ± standard deviation of duplicate analyses; nd, not detected.<sup>b</sup> Day post-partum ± 2 h.<sup>c</sup> NT, nucleotide.

**Table 3.**Significance levels for rates of decrease of nucleosides and nucleotides (NT) in bovine milk <sup>a</sup>.

Form	Cytidine	Uridine	Guanosine	Adenosine	Total Form
Seasonal differences (winter vs. summer) between slopes: p-values <sup>b</sup>					
Nucleoside	< 0.001	< 0.001	< 0.001	0.600	< 0.001
Monomeric NT	0.310	< 0.001	< 0.001	0.007	< 0.001
NT Adduct	0.048	< 0.001	< 0.001	0.002	< 0.001
Polymeric NT	0.303	0.002	< 0.001	< 0.001	< 0.001
Total Base	0.676	< 0.001	< 0.001	0.107	< 0.001
Non-zero slope (summer): p-values <sup>c</sup>					
Nucleoside	< 0.001	< 0.001	< 0.001	1.000	< 0.001
Monomeric NT	0.168	0.182	0.384	0.002	0.207
NT Adduct	0.437	0.051	0.097	0.552	0.030
Polymeric NT	0.196	0.233	< 0.001	0.001	< 0.001
Total Base	0.769	< 0.001	< 0.001	0.386	< 0.001
Non-zero slope (winter): p-values <sup>c</sup>					
Nucleoside	< 0.001	< 0.001	0.048	0.316	< 0.001
Monomeric NT	0.511	< 0.001	< 0.001	0.905	< 0.001
NT Adduct	0.019	< 0.001	< 0.001	0.001	< 0.001
Polymeric NT	0.399	0.001	< 0.001	< 0.001	< 0.001
Total Base	0.408	< 0.001	< 0.001	0.053	< 0.001

<sup>a</sup> Level of significance  $P \leq 0.05$ .<sup>b</sup> Statistical significance means there is evidence that there is a real difference between seasons.<sup>c</sup> Statistical significance means there is evidence that the levels are actually decreasing.

**Fig. 1.** Chromatograms of a mixed nucleoside standard and colostrum sample. Conditions: mobile phase A: 0.05 M sodium acetate, pH = 4.8; mobile phase B: 100 % methanol; gradient elution: flow rate 0.7 mL min<sup>-1</sup> throughout, 0–3 min (95 % A, 5 B v/v), 7–22 min (75 % A, 25 B v/v), 23–30 min (95 % A, 5 B v/v). UV detection 260 nm.

**Fig. 2.** Total potentially available nucleosides in pooled bovine milk samples from winter-milk (A, B) and summer-milk (C, D) herds over the first month of lactation. A, C: ☒, cytidine; ☐, uridine; ☑, guanosine; ☐, adenosine. B, D: ☐, polymeric nucleotides; ☒, nucleotide adducts; ☐, monomeric nucleotides; ☑, nucleosides.

**Fig. 3.** Percentage contribution to total potentially available nucleosides in pooled bovine milk samples from winter-milk (A, B) and summer-milk (C, D) herds over the first month of lactation. A, C: ☒, cytidine; ☐, uridine; ☑, guanosine; ☐, adenosine. B, D: ☐, polymeric nucleotides; ☒, nucleotide adducts; ☐, monomeric nucleotides; ☑, nucleosides.

