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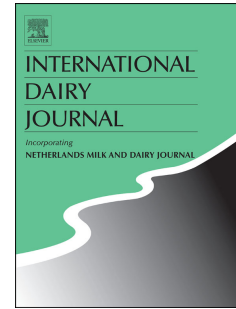
1 **Determination of total potentially available nucleosides in bovine milk**

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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 _____

1. Introduction

30

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 β-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, Barness, & 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 μL

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_{18} column, 5 μm, 4.6 × 150 mm

113 (Phenomenex, Torrance, CA, USA).   Prior to use, mobile phases were filtered

114 and degassed using a filtration apparatus with 0.45 μm 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, β-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 ≥ 18 MΩ 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 ºC, 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 pH = 7.35 ± 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 μg, 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 (pH = 8.5,   3 h), which dephosphorylated monomeric nucleotides to

168 nucleosides. The third treatment incorporated nuclease (pH = 5.1, 16 h) and

169 phosphatase (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 (pH = 5.1, 16 h),

172 pyrophosphatase and phosphatase (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_{18} 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^{-1} 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 (95.0–135.0 µg 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 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 ± standard deviation) through the

251 affinity gel clean-up were as follows: cytidine (93.4 ± 1.1%), uridine

252 (92.3 ± 5.1%), guanosine (88.3 ± 4.9%), adenosine (95.2 ± 4.2%), and

253 5-methylcytidine (92.6 ± 2.3%). Recoveries measured through the enzymatic

254 digestion and subsequent affinity gel clean-up were: cytidine (95.5 ± 2.8%),

255 uridine (101.7 ± 3.7%), guanosine (89.2 ± 2.4%) and adenosine (94.7 ± 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 ~50 µmol 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 1–3 µmol 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 colostral

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 µmol 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.,

322   1995; Van Buren, Kulkarni, Fanslow, & Rudolph, 1985) and, more recently,

323   Mashiko et al. (2009) demonstrated that dietary UMP affected the immune

324   response of newborn calves.

325

326   *3.3.3. Nucleotide adduct contribution to TPAN*

327   The results for uridine adducts in the present study ranged from not

328   detected to 23.7 μmol dL^{-1} in the winter-milk herd and from not detected to

329   6.8 μmol dL^{-1} in the summer-milk herd, with a rapid reduction in concentration

330   after the third day post-partum. Guanosine adducts measured ranged from

331   not detected to 3.9 μmol dL^{-1} in the winter-milk herd and from not detected to

332   1.2 μmol dL^{-1} in the summer-milk herd. Similar levels of adenosine adducts

333   were found, presumably derived from flavin adenine dinucleotide and

334   nicotinamide adenine dinucleotide (Fox & McSweeney, 1998; Kanno,

335   Shirahuji, & Hoshi, 1991). Utilising enzymatic techniques, Gil and Sánchez-

336   Medina (1981) measured UDP hexosamine, UDP hexose and UDP galactose

337   concentrations in bovine colostrum and milk, which ranged from not detected

338   to ~104 μmol dL^{-1}. Levels were highest at 27 and 78 h and much lower or

339   absent in subsequent stages of lactation. Guanosine diphosphate fucose was

340   also reported at 27 and 78 h, at levels of 6.7 and 4.1 μmol dL^{-1}, respectively.

341

342   *3.3.4. Polymeric nucleotide contribution to TPAN*

343   The concentration of polymeric nucleotides in bovine colostrum was

344   similar to that in human colostrum and milk, however, with advancing lactation,

345   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.

434 **Acknowledgements**

435

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Table 1.

Nucleosides and nucleotides in bovine milk from a winter-milk herd (μmol dL^{-1})[a].

Day[b]	Form[c]	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

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

Table 2.

Nucleosides and nucleotides in bovine milk from a summer-milk herd (μmol dL^{-1}) [a].

Day[b]	Form[c]	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

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

Table 3.

Significance levels for rates of decrease of nucleosides and nucleotides (NT) in bovine milk [a].

Form	Cytidine	Uridine	Guanosine	Adenosine	Total Form
Seasonal differences (winter vs. summer) between slopes: p-values[b]					
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[c]					
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[c]					
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

[a] Level of significance $P <= 0.05$.
[b] Statistical significance means there is evidence that there is a real difference between seasons.
[c] 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^{-1} 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.