1 7238

2 **Exploring the use of tertiary reclaimed water in dairy cattle production**

3

4 Marta Terré[a*], Carme Bosch[b], Alex Bach[a,c], Francesc Fàbregas[a], Carme Biel[d], Marta

5 Rusiñol[e], Silvia Bofill[e], Montse Calderer[b], Irene Jubany[b], Xavier Martínez-Lladó[b],

6 [a] Department of Ruminant Production, Research and Technology Agrifood Institute

7 08140 Caldes de Montbui, Barcelona, Spain

8 [b] Fundació CTM Centre Tecnològic, 08243 Manresa, Barcelona, Spain

9 [c] ICREA, Institució Catalana de Recerca i Estudis Avançats, 08007, Barcelona, Spain

10 [d] Department of Environmental Horticulture, Research and Technology Agrifood

11 Institute 08140 Caldes de Montbui, Barcelona, Spain

12 [e] Laboratory of Viruses Contaminants of Water and Food, Department of Genetics,

13 Microbiology and Statistics, Faculty of Biology, University of Barcelona 08028

14 Barcelona, Spain

15

16

17

18

19

20 [*]corresponding author: Marta Terré

21 Email: marta.terre@irta.cat

22 Declaration of interests: none

23

24

25

26 **Abstract**

27 The objective of this study was to explore through both *in vitro* and *in vivo* experiments

28 the use of reclaimed urban wastewater in dairy cattle production systems with the aim

29 of improving water efficiency and sustainability. Firstly, the use of different tertiary

30 treatments (ultrafiltration (UF), ultraviolet disinfection (UV), chlorination process, and

31 their combination) to improve the quality of an urban secondary effluent was studied in

32 intestinal primary cell cultures evaluating the expression of genes related to apoptosis,

33 cell damage, and inflammation. The results revealed that secondary treated wastewater

34 and waters that were treated with a chlorination process (even tap water) caused an

35 increase in apoptosis, intestinal primary cell damage, and inflammation. The *in vivo*

36 experiment evaluated the short-term effects on health and performance of using UF- and

37 UV-treated secondary effluent compared with the use of tap water for drinking and

38 preparing milk replacer in young calves from 5 to 47 days of age. Calves previously fed

39 with UF+UV treated secondary effluent clearly preferred tap water when they were

40 exposed to a double water choice at the end of the study. This reduction of the

41 palatability and acceptability was probably due to a greater level of water salinity of the

42 treated reclaimed water (570 vs 1,437 ± 76.5 μS/cm of conductivity for tap water and

43 UF-UV treated secondary effluent, respectively), which potentially entailed a reduction

44 of calf concentrate intake (466 vs 351 ± 32.2 g/d for calves fed with tap water and UF-

45 UV treated water, respectively). The use of reclaimed water did not pose an acute risk to

46 animal health. It is concluded that improvements on the tertiary treatment to reduce

47 water salinity should be considered when using reclaimed water for drinking purposes

48 in livestock production systems. This study is a first approach to a more sustainable and

49 efficient use of water in animal husbandry for countries with water scarcity. However,

2

50 more studies are required before its implementation to further study long-term effects

51 and the presence of new-contaminants not defined in the current legislation.

52 Keywords: livestock drinking water; reclaimed water; ultrafiltration; water reuse

53 **Abbreviation list**

54 ACTB: β-actin

55 BNIP3: Adenovirus E1B 19 kDa protein-interacting protein 3

56 CASP3: Caspase 3

57 DM: dry matter

58 HSPA1A: Heat shock 70 kDa protein 1A

59 HSPB1: Heat shock protein family B member 1

60 IL-1ß: Interleukin 1 beta

61 IL-10: Interleukin 10

62 MR: Milk replacer

63 TDS: total dissolved solids

64 TIC: total inorganic carbon

65 TNF-α : Tumor necrosis factor alpha

66 TOC: total organic carbon

67 TSS: total suspended solids

68 UF: ultrafiltration

69 UV: ultraviolet

70 WW: wastewater

71 **1. Introduction**

72 The agricultural sector accounts for around 70% of water use worldwide, and it remains

73 one of the major sources of water pollution with fertilizer run-off, pesticide, and

74 livestock effluents (FAO, 2015). Furthermore, the prediction of rising world human

75 population will increase water demand in this sector (Gulbenkian Think Tank, 2014),

76 and future policies must look for more sustainable food production systems to avoid

77 serious food and water crisis in the upcoming years. Water reduction and sustainable

78 systems in crop production, such as more efficient irrigation systems (Kusakabe et al.,

79 2016; Singh et al., 2016) or crops more adapted to drought areas (Daryanto et al., 2016;

80 Vurukonda et al., 2016) merit current attention. Within the primary sector, it is

81 estimated that livestock production (including irrigation of grains, forages, and pastures

82 plus water usage for animal husbandry) uses 29% of the total agriculture water demand

83 (Mekonnen and Hoekstra, 2012). However, much less attention has been paid to the

84 livestock production system regarding efficiency of water utilisation (Ran et al., 2016).

85 To face the increasing water scarcity and water pollution, some initiatives involving

86 water reuse, mainly from urban sources, have been implemented in some countries

87 (Kihila et al., 2014) as an economically-feasible method of increasing existing water

88 supply, especially when compared with expensive alternatives such as desalination or

89 development of new water sources involving dams and reservoirs (Shannon et al.,

90 2008). The most common water reuse application in the agricultural sector involves

91 irrigation of food crops, pastures, and industrial non-food crops (Maestre-Valero et al.,

92 2016; Jiang et al., 2016), and to a lesser extent aquaculture (Feldlite et al., 2008), and

93 silviculture (House et al., 1999). In the livestock sector either groundwater or surface

94 water is used to supply water to animals depending on their locations. However, only

95 the Environment Protection Authority of Victoria in Australia has regulated the

96 implementation of reclaimed water usage in animal husbandry. The two main water

97 uses in intensive dairy cattle production systems are for drinking (82%) and cleaning

98 farm facilities (18%) (Drastig et al., 2010), and these needs are fairly constant

99 throughout the year. In contrast, for crops irrigation water demand is seasonally, which

4

100 makes the implementation of more reclaimed water systems difficult. In general, quality

101 requirements for livestock drinking water and their impact on livestock health and

102 performance are poorly investigated. There are no specific legal requirements

103 concerning quality of drinking water for dairy cattle (in most of the legislation is

104 mentioned suitable and healthy water), with most documents being mere guidelines

105 from governmental and academic institutions (South Africa Department of Water

106 Affaires and Forestry, 1996; Olkowski, 2009; Schlink, 2010; Department of

107 Environment, Food and Rural Affairs of United Kingdom, 2012; Department of

108 Primary Industries and Regional Development of Australia, 2017).

109 The motivations of the present study were the need for new strategies to improve water

110 efficiency and sustainability in livestock production coupled with the availability of

111 economical reasonable water treatment technologies that apparently result in sufficient

112 water quality that could have no negative effects on health and productivity of dairy

113 cattle. Therefore, the two main objectives of the study were: 1) to evaluate, in an *in vitro*

114 system, the most suitable tertiary wastewater (WW) treatment process to obtain

115 reclaimed water of sufficient quality for dairy cattle drinking purposes, and 2) to

116 evaluate in an *in vivo* study the short-term effects on health and performance of offering

117 reclaimed water to dairy calves.

118 **2. Materials and methods**

119 *2.1. In vitro study*

120 This study was performed in the facilities of IRTA in Torre Marimon (Caldes de

121 Montbui, Spain), and WW was obtained from the urban WW treatment plant in Caldes

122 de Montbui (Barcelona, Spain.), which received mostly municipal discharges. This

123 plant was situated at approximately 1 km from IRTA facilities. Water treatment in this

5

124 urban WW treatment plant includes a physicochemical primary treatment followed by

125 biological and settling secondary treatments.

2.1.1. Tertiary wastewater treatment selection

127 The WW (composition depicted in Table 1) intended for tertiary reclamation was the

128 effluent from the secondary treatment of the WW treatment plant. The water tertiary

129 treatment to obtain water of suitable quality for calves drinking should pursue a

130 reduction in the microbiological load and a reduction of water turbidity, following the

131 Australian guidelines for the use of reclaimed water (Class B, pH 6-9, < 100 cfu of

132 *Escherichia coli* /100 mL, < 20 mg/L Biological Oxygen Demand, < 30 mg/L

133 suspended solids, and a reduction of helminth eggs). In the present study, several

134 tertiary treatments were initially tested in intestinal primary cell cultures to select the

135 most suitable for conducting a subsequent *in vivo* study with dairy calves. The tertiary

136 treatments consisted of a combination of several technologies: ultrafiltration (UF) with a

137 30 nm pore membrane (66.03 I8 Berhof Membrane Technology GmbH, Eningen,

138 Spain); ultraviolet (UV) disinfection (STERILUX MINI-1000 CEASA, Castellví de

139 Rosanes, Barcelona, Spain) with a targeted UV dose of 80 mJ/cm^2 (UV); and

140 chlorination with addition of sodium hypochlorite to achieve 1 mg/L of free chlorine

141 (ClO$^-$).

142 *2.1.2. Intestinal primary cell cultures*

143 Jejunum tissue was obtained at a slaughterhouse from an 11-mo old bull and

144 immediately transported in chilled phosphate-buffered saline with 100 µg/mL

145 streptomycin, 100 U/mL penicillin, and 2.5 µg/mL amphotericin B to the laboratory. In

146 the laboratory, tissue was cut into small pieces and washed in phosphate-buffered saline

147 with 0.1 mM ethylenediaminetetraacetic acid and 0.1 mM dithiothreitol for 10 min at

148 37ºC in 5% CO_2 at 150 rpm. Then, supernatant was removed and Roswell Park

6

149 Memorial Institute (RPMI) 1640 media with 0.25% collagenase was added and

150 incubated for 15 min at 37ºC in 5% CO_2 at 150 rpm. The supernatant containing

151 isolated epithelial cells was added to a same volume of RPMI 1640 media with 0.02

152 mg/mL DNase. This step was repeated 3 times. Then, supernatants (containing the cells)

153 were centrifuged at 300 g for 5 min and the cell pellets resuspended in Dulbecco's

154 Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) media with 8 µg/mL

155 bovine insulin, 10 µg/mL gentamycin, 50 µg/mL hydrocortisone, 10% fetal bovine

156 serum, 100 µg/mL streptomycin, 100 U/mL penicillin, and 2.5 µg/mL amphotericin.

157 Cells were quantified by microscopy and incubated at 80,000 cells/cm^2 in 175 cm^2

158 flasks for 24 h at 37ºC in 5% CO_2 at 150 rpm. Epithelial cell phenotype was confirmed

159 by immunofluorescence staining against anti-cytokeratin antibodies (Sigma-Aldrich,

160 Saint Louis, US).

161 *2.1.3. Primary cell culture*

162 Jejunum cells were cultured in 24-well plates at 37ºC under a 5% CO_2 atmosphere, at a

163 cell density of 50,000 cells/well during 16 h. Then, cells were treated during 2 h with

164 eight different types of water: 1) cell media culture (MC) as a negative control to

165 evaluate the effects of incubating the intestinal cells in the plates, 2) tap water (TW) to

166 evaluate a potable water source, 3) water from a drinker from a dairy farm filled with

167 chlorinated groundwater (ThW) to evaluate the water that animals are consuming in the

168 field conditions, 4) secondary effluent of the WW treatment plant (SW) to evaluate the

169 improvements of the tertiary treatments proposed in the study, 5) secondary effluent

170 with an UF treatment (UF), 6) secondary effluent with an UV disinfection (UV), 7)

171 secondary effluent with UF and UV treatments (UF+UV), and 8) secondary effluent

172 with UF and chlorination (UF+ClO$^-$). After the incubation, cells were washed and lysed

173 with TriZol (Invitrogen, Paisley, UK) to extract RNA and quantify, by qPCR,

7

174 expression of apoptotic (*BNIP3* and *CASP3*), cell damage (*HSPA1A* and *HSPB1*), and

175 inflammation (*TNF α* , *IL-1 β* , and *IL-10*) genes.

176 *2.1.4. Sampling and analyses*

177 Five-litres water samples were obtained in a plastic container in the wastewater

178 treatment plant, and within the same day they were sent refrigerated (5-7 ℃) to the

179 laboratory. Samples were kept refrigerated in the laboratory until the different

180 treatments to produce the studied waters were applied. Samples of the different

181 treatments were obtained to analyse them for pH, conductivity, turbidity, chemical

182 oxygen demand (COD), ammonium (NH_4^+), chloride (Cl^-), phosphate (PO_4^{3-}), nitrate

183 (NO_3^-), sulphate (SO_4^{2-}), aerobic bacteria counts, and *E. coli* counts following analytical

184 standard methods for water quality. COD was determined by the method 5220 defined

185 in the Standard Methods for the Examination of Water and Wastewater (1998) and

186 anions and cations were analysed by ionic chromatography (Dionex ICS-2100).

187 Microbiological characterisation was performed following the standard methods UNE-

188 EN ISO 6222 and UNE-EN ISO 9308‐1 for aerobic counts and *E. coli*, respectively.

189 Total RNA from the cells was extracted using Trizol (Invitrogen, Paisley, UK). One

190 microgram of RNA was retrotranscribed to cDNA using IScript cDNA synthesis kit

191 (Bio-Rad, Hercules, CA) following the manufacturer's instructions. Real-time PCR was

192 performed using specific primers described in Table 2. A total reaction volume of 20 µL

193 containing 100 ng of cDNA, 10 µL SYBR Green (Bio-Rad Laboratories) was used at

194 the optimized primer concentration for each gene (Table 2). The relative expression of

195 selected genes was calculated using the delta cycle threshold (ΔCt) method with ß-actin

196 (*ACTB*) as the reference gene, and a randomly-chosen sample of the media culture

197 treatment as a calibrator following Pfaf (2014).

198 *2.1.5. Statistical analysis*

8

199 An analysis of variance with the type of water as the main effect was performed for all

200 data. Outcome variables that did not follow a normal distribution were log-transformed.

201 Least square means and the standard error of the mean (SEM) presented herein

202 correspond to non-transformed data, and the *P*-values correspond to the results with the

203 log-transformed model. Significance was declared at P<0.05 and tendencies at ≤0.10,

204 using the Fisher's protected LSD test to assess differences among treatments.

205 *2.2. In vivo study*

206 *2.2.1. Tertiary reclaimed water production*

207 The secondary effluent from the wastewater treatment plant was transported by a tanker

208 truck to a 27 m^3 closed tank in the facilities of IRTA. The onsite system for the tertiary

209 treatment consisted of a UF and a UV disinfection process. The UF consisted of a

210 HyperFlux tubular module (model 66.03 I8, Berghof Membrane Technology GmbH,

211 Eningen, Germany) and was operated in crossflow mode to have around 300L/d of

212 permeate. All piping was made of plastic black tubing to avoid algae growth. The

213 system worked intermittently and started/stopped automatically following a set program

214 (running from 3:00 to 6:00 a.m., from 7:00 to 10:00 a.m., and from 10:00 a.m. to 1:00

215 p.m.) to avoid overheating of the circulation pump. The UF system consisted of some

216 programmable logic controllers with programs and security sensors (i.e., water level).

217 To check the working pressure and pressure drop during filtration manometers were

218 installed at the intake and outtake of the UF module. Permeate was diverted to a black

219 storage tank of 1,000 L, which fed the UV module. The UV operated daily at a flow rate

220 of 300 L/h (UV dose of 80 mJ/cm^2) to obtain the amount of reclaimed water required

221 for the preparation of milk replacer (MR) and water drinking for 10 calves during the

222 study.

223 *2.2.2. Animals and treatments*

224 Eighteen Holstein dairy calves of 5 ± 3.2 d of age and 40 ± 6.3 kg of body weight were

225 gathered from several farms, and raised at the facilities of IRTA according to the

226 recommendations of the animal Care Committee of IRTA. Calves were housed

227 individually and bedded with sawdust. Two different types of MR feeding programs

228 were tested in order to achieve different amounts of reclaimed water consumption by

229 the animals (as calves would consume much larger quantities of MR than water alone).

230 The experiment followed a 2x2 factorial design, two different MR feeding programs (4

231 vs 8 L/d of MR at 12.5% dry matter (DM) throughout the study), and two different

232 water sources (TW vs UF+UV) that calves consumed through both MR feeding and

233 drinking water. Concentrate and barley straw were offered *ad libitum* from the

234 beginning of the study until calves reached 47 d of age (study end).

235 *2.2.3. Sampling and analysis*

236 Calves were weighed at the beginning of the study and once weekly thereafter. Milk

237 replacer, concentrate, straw and water intakes were measured daily, and veterinary

238 treatments were recorded. Calf faecal consistency was evaluated daily using a 3-point

239 scale (1: normal, 2: loose, 3: watery). Blood samples were obtained at the beginning and

240 at 35 d of study to determine glucose, insulin, urea, creatinine, hepatic enzymes (AST

241 and GGT), non-esterified fatty acids (NEFA), triglycerides (TG), and thyroid hormone

242 (T_3) serum concentrations, and conduct a full haematological profile. Faecal samples

243 were also obtained at the beginning and at 35 d of study to assess the presence of

244 helminthic eggs, *Cryptosporidium* cysts, and coccidia oocysts.

245 The last day of study, a preference test was performed to evaluate the capacity of

246 animals to distinguish between TW and UF+UV waters and to determine whether

247 calves had a preference for any of them. During that day, all animals were offered the

248 two types of water (TW and UF+UV) in two separate buckets to drink. At 9:00 a.m. all

10

249 calves were offered 5,500 mL of each type of water, then at 1:30 p.m. water

250 consumption up to that point was recorded and additional water was offered if the

251 buckets contained less than 4,000 mL (consumption >1,500 mL). At 4:30 p.m. water

252 consumption was determined again (by weighing the buckets) and the water leftover

253 was completely replaced with new water until 9:00 a.m. of the following day, when all

254 buckets were weighed again to assess water consumption.

255 During the study, water samples of secondary water and the effluents after each

256 treatment step (UF effluent, storage tank before UV, UV effluent, and water from both

257 TW and UF+UV calf buckets) were collected fortnightly to determine water pH,

258 conductivity, turbidity, total organic carbon (TOC), total suspended solids (TSS), and

259 counts of aerobic bacteria, *Clostridium perfringens*, total coliforms, and *E. coli.* In

260 addition, total inorganic carbon (TIC), total dissolved solids (TDS), and concentration

261 of anions, cations, total Mn, and Fe, and contents of several toxic organic and inorganic

262 trace constituents including pesticides, halogenated toxic compounds, polycyclic

263 aromatic hydrocarbons, nonylphenols, and heavy metals were determined in reclaimed

264 water (UF+UV) and TW at least once throughout the duration of the study, following

265 standard analytical methods. Water samples were collected in different containers

266 depending on the parameter to be analysed: for the determination of organic

267 contaminants samples were kept in ambar glass bottles, for the microbiological

268 parameters in sterile plastic containers and for the remaining parameters in plastic.

269 Samples were sent refrigerated to the laboratories and kept refrigerated until analysis.

270 Briefly, turbidity, TSS, and TDS were determined following methods 2130D, 2540G,

271 and 2540C from the Standard Methods for the Examination of Water and Wastewater

272 (1998), respectively. Water was cultivated at 37°C during 24 h in plate count agar

273 media, and in chromogenic media Compact Dry EC (Hardy Diagnostics, Santa Maria,

11

274 CA) to determine counts of aerobic, coliforms, and *E. coli*, respectively. TOC and TIC

275 were analysed using a total carbon analyser (Analytik-Jena 3100 N/C). Metal

276 concentrations in water were determined by inductively coupled plasma mass

277 spectrometry (7500 CX, Agilent), and analyses of organic constituents were determined

278 by high-resolution gas chromatography coupled to mass spectrometry.

279 Reclaimed water samples were analysed three times during the study for potential

280 microbiological risks. Reference indicators for microbial hazards determined in the

281 present study were: bovine polyomaviruses (as indicator of bovine faecal

282 contamination), human adenoviruses (a human fecal viral indicator), somatic

283 coliphages, *Clostridium perfringens*, *Cryptosporidium spp.*, and helminth and *Taenia*

284 *spp.* Eggs. For virus detection, 10 L of reclaimed water samples were concentrated by

285 skimmed milk flocculation (Calgua et al., 2013) while wastewater samples were

286 concentrated by utracentrifugation (Pina et al., 1998) and viral nucleic acids were

287 extracted using the QIAmp Viral RNA kit (QIAgen, Inc.) to further quantify human

288 adenoviruses and bovine polyomaviruses by qPCR (Hernroth et al., 2002; Hundesa et

289 al., 2010). For *Cryptosporidium*, one litre of water was filtered using cellulose acetate

290 filters of 0.2 μm pore diameter (Whatman, GE Healthcare, Germany), and DNA was

291 extracted using the DNeasy blood and tissue kit (QIAgen, Hilden, Germany) to further

292 quantify by qPCR *Cryptosporidium* following Guy et al. (2003). To detect *Clostridium*

293 *perfringens*, water samples were cultured in the selective media tryptone sulphite

294 neomycin agar during 24 h at 37°C in an anaerobiosis jar with Anaerocult A (Merck) to

295 consume the oxigen. Somatic coliphages were determined by incubating 100 μL of a

296 dilution of the tested water in BBL^{TM} media and *E.coli* BL21 on Luria-Bertani media,

297 and monitoring plaque formation after 8-12 h of incubation at 37˚C. Lastly, 10 L of

298 water were used to detect by optical microscopy the presence of eggs from intestinal

299 nematodes and *Taenia spp.*.

2.2.4. Statistical analysis

301 Data pertaining to growth performance and feed intake were analyzed with a mixed-

302 effects model with repeated measures, including the fixed effects of milk-feeding

303 program, type of water, week of study and their 2- and 3-way interactions, plus the

304 random effect of calf. Initial body weight, initial age, and farm of origin were used as

305 covariates in the model, and week entered the model as a repeated measure using an

306 autoregressive covariance matrix.

307 The incidence of scours was analyzed with a mixed-effects logistic regression being the

308 proportions of observation of score 2 the independent variable. The model considered

309 milk-feeding program, type of water, and their interaction as fixed effects.

310 Hematological and blood biochemical profiles were analyzed with an analysis of

311 variance including the effects of milk-feeding program, type of water, and their 2-way

312 interactions plus initial age and initial values (day 0 of study) as covariates. Parameters

313 that did not follow a normal distribution were log-transformed. Least square means and

314 SEM presented herein correspond to non-transformed data, and *P*-values correspond to

315 the results with the log-transformed model. Significance was declared at $P < 0.05$ and

316 tendencies at $P \leq 0.10$.

317 Water preferences were determined as a preference ratio for TW as follows:

318 $$\text{Preference ratio} = (\text{TW intake})/(\text{TW intake} + (\text{UF+UV}) \text{ intake})$$

319 Values for preference ratio greater than 0.5 indicate a preference for TW, values equal

320 to 0.5 denote no preference for any type of water, and preference ratio values lower than

321 0.5 indicate a preference for UF+UV water.

13

322 Preference ratios for all calves (independently of their previous water experience) were

323 analyzed for a difference from 0.5 (lack of preference) using a t-test. Then, to check the

324 effect of the previous exposure to different water sources, preference ratio for TW

325 throughout the 24-h were also analyzed using a mixed-effects model considering type of

326 milk feeding program, type of water, and their 2-way interaction as fixed effects.

3. Results and discussion

3.1. In vitro study

329 The present *in vitro* experiment explored the effects that different tertiary water

330 treatments applied on a secondary effluent from a municipal wastewater treatment plant

331 had when they came into contact with bovine intestinal cells. Chemical and

332 microbiological quality of the different water types and treatments are shown in Table

333 1. Intestinal primary cells cultured with TW, ThW, SW, UV, and UF+ClO$^-$ had an

334 increased ($P < 0.05$) expression of *BNIP3*, *HSPA1A*, *TNF-α*, and *IL-10* genes

335 compared with those cultured with MC, UF, and UF+UV (Figure 1), denoting an

336 increase of cellular apoptosis, cell damage, and inflammation. Although the

337 experimental design does not allow determining which chemical or microbial

338 parameters were responsible of the differences in gene expression, some hypothesis can

339 be made to explain the results herein. Interestingly, TW, ThW, and UF+ClO$^-$ had in

340 common a chlorination process and this may be, in part, the cause of the negative

341 impacts on intestinal primary cells. It has been previously described that chlorine

342 disinfection by-products can cause cellular oxidative stress, and they may have

343 carcinogenic and mutagenic properties (Yuan, et al., 2006; Richardson, et al., 2007). On

344 the other hand, waters that achieved similar results than those obtained with MC

345 (considered the optimum media for intestinal cells) and had the lowest impact on

346 intestinal primary cells, were the treatments including a UF process. The UF treatment

14

347 was intended to eliminate suspended particles and colloids and partly reduce the

348 microbiological load. It was not possible to associate the low gene expression elicited

349 by the UF treatment with an elimination of the microbial load because UV disinfection

350 alone did not generate similar results to MC. Thus, perhaps the good performance of UF

351 could be associated to the elimination of toxic constituents attached to suspended

352 particles (e.g., polycyclic aromatic hydrocarbons, heavy metals), which should have

353 been removed through the UF process (Smol et al., 2012). To our knowledge, this is the

354 first study that clearly indicates that the least harmful water for intestinal cells was the

355 reclaimed water treated by UF and UV. Therefore, UF treatment followed by UV

356 disinfection was the tertiary treatment selected to offer to calves in the *in vivo* study.

357 *3.2. In vivo study*

358 The *in vivo* study evaluated the short-term effects on performance and health of feeding

359 dairy calves with a tertiary treated effluent from a wastewater treatment plant or tap

360 water. The tertiary treatment consisted of an UF needed to reduce part of the microbial

361 load such as parasite eggs and spores that are not removed with an UV disinfection

362 needed to eliminate other microbial hazards such as bacteria and viruses. The

363 combination of both techniques allowed having a multi-barrier treatment. Reclaimed

364 water quality achieved by the proposed UF+UV reuse scheme fulfilled the water quality

365 objectives proposed by the Australian guidelines (Environment Protection Authorities

366 of Victoria, 2003) for the use of reclaimed water for livestock drinking in ruminants.

367 Physicochemical characterization of reclaimed water in comparison to TW is shown in

368 Table 3. Levels of toxic inorganic and organic constituents in reclaimed water were low

369 and fulfilled water guidelines for both human and livestock drinking water (Schlink,

370 2010; Olkowski, 2009; Spanish RD 140/2003). However, the concentration of certain

15

371 ions, such as K^+, Na^+, PO_4^{3-}, and Cl^-, slightly exceeded the upper levels indicated in

372 Schlink et al. (2010).

373 There was no interaction between MR feeding program and type of water on

374 performance and feed intake parameters (Table 4). Calves fed 8L of MR had a greater

375 growth performance ($P < 0.05$), reduced concentrate intake ($P < 0.001$), and improved

376 feed efficiency ($P < 0.001$) in comparison with those fed 4L, independently of the type

377 of water offered. This effect was expected because there is a negative relationship

378 between the amount of MR offered to calves and concentrate intake (Terré et al., 2009),

379 and as MR is more digestible than concentrate, feed efficiency of calves improves when

380 more MR is offered. Calves that were offered UF+UV consumed less concentrate than

381 TW-fed calves ($P < 0.05$). There exists a close relationship between water and

382 concentrate intake (Kertz et al., 1984), and low availability of water is usually related to

383 a decrease in calf starter concentrate intake. However, water intake was similar in both

384 treatments. Constituents associated with salinity may affect water acceptability and

385 palatability and livestock performance. Generally, increasing salt content in water for

386 dairy cows decreases water and feed intake, and milk yield (Challis et al., 1987;

387 Solomon et al., 1995).

388 Regarding the microbiological quality, obtained UF+UV water did not present any

389 potential microbial risk (Table 3), and all reference microbiological indicators were

390 below threshold limits. Sewage samples obtained from the wastewater treatment plant

391 used for wastewater collection were evaluated for the potential presence of bovine fecal

392 contamination by analysing bovine adenoviruses. Since, bovine adenoviruses were not

393 detected; the probability of bovine fecal contamination of the wastewater used to

394 produce reclaimed water for calves drinking purposes was low. These results were in

395 concordance with the lack of bovine exploitations or slaughterhouses in the area

surrounding the wastewater treatment plant evaluated. For this reason, human adenoviruses that are present in all urban sewage samples, being excreted persistently by human population, were used as indicators of the efficiency of the UF+UV treatment applied. Since they are extremely stable to UV inactivation, they represent ideal indicators of UV viral inactivation. It is well known that the absence of bacteria indicators do not correlate with the absence of viruses so it is relevant to test reclaimed water used for drinking purposes for the presence of viruses. Since viruses are host-specific microorganisms, bovine adenoviruses may be used as indicators of bovine fecal contamination while other animal viruses have been described to indicate animal fecal contamination from other sources (Bofill-Mas et al., 2013).

Total aerobic counts increased 3-log cfu/mL in the storage UF tank after the UF treatment, but after the UV treatment a 5-log cfu/mL reduction was observed in the reclaimed water ($P < 0.05$). Loss of water quality occurred in some steps of the treatment scheme because the treatment did not work continuously and some aerobic bacteria re-growth occurred during storage time in the tanks. However, the UV treatment was able to reduce bacterial counts again before water was used to prepare MR and also offered to calves as drinking water (Figure 2). The regrowth of bacteria in reclaimed water after storage has been described elsewhere (Jjemba et al., 2010; Li et al., 2013). Bacteria regrowth during the storage step in the present study could be envisaged because UF+UV water contained organic carbon and phosphorous, which are the two main limiting nutrients needed for microbial growth (Table 3).

Faecal score was measured in calves fed with UF+UV and TW as indicator of faeces consistency and assess incidence of diarrhoea. There were no differences in the probability of faecal score of 2 in any of the treatments, and the number of veterinary

17

420 treatments against diarrhoea and respiratory problems was similar between UF+UV and

421 TW calves.

422 Haematological and biochemical parameters were measured in all animals to detect

423 possible variations in general health and metabolic status. Haematological blood

424 profiles were similar between animals fed TW and UF+UV either at 4 or 8 L/d, with the

425 exception of blood eosinophil and platelet counts. Blood eosinophil counts tended (P =

426 0.08) to be greater in calves reared with UF+UV than those offered TW (0.61 vs 0.24 ±

427 0.163, 10^3/µL, respectively), and platelet counts tended (P = 0.05) to be lower in

428 UF+UV than in TW calves (605 vs 766 ± 49.8, 10^3/µL, respectively). Most of the

429 haematological parameters were in the range reported elsewhere (Knowles et al., 2000;

430 Brun-Hansen et al. 2006). However, blood eosinophil counts in the present study were

431 within physiological ranges of adult cattle (Roland et al., 2014), but in greater

432 concentration than in those observed in calves (Knowles et al., 2000; Brun-Hansen et al.

433 2006). Conditions commonly associated with eosinophilia included hypersensitivity

434 reactions and parasitic infections. Additional causes are neoplasia, infections, and drug

435 reactions (Roland et al., 2014). In the present study, faeces of calves were checked for

436 coccidia oocysts, nematodes eggs, and the presence of *Cryptosporidium* cysts. Only

437 *Cryptosporidium* cysts were found at the beginning of the study in both treatments (P =

438 0.34; 44.4 vs 55.6 % of positive calves were distributed in the UF+UV and TW,

439 respectively), but no helminth eggs or cysts were detected at 35 d of study in any of the

440 treatments. Therefore, either parasitic infections other than those caused by coccidia,

441 nematodes or cryptosporidia occurred in UF+UV calves to explain the deviations on

442 blood eosinophil and platelets counts or, the presence of toxic constituents in the

443 secondary effluent, which may not have been eliminated by the UF treatment, could be

444 the 2 hypothetical explanations for the observed haematological variations. In any of the

445 cases, the problem should be considered as mild, since a greater infection or problem

446 would have changed more parameters of the haematological profile.

447 The biochemical blood profile presented some differences among treatments: serum

448 GGT concentrations were lower ($P < 0.05$) in 8-L than in 4-L fed calves, serum NEFA

449 concentration lower ($P < 0.05$) in UF+UV than in TW fed calves, serum TG

450 concentrations were lower ($P < 0.05$) in 4L-TW and 8L-UF+UV than in 8L-TW and

451 4L-UF+UV, and serum thyroid hormone concentrations were greater ($P < 0.01$) in 8-L

452 than in 4-L calves. Blood glucose and insulin are related to carbohydrate metabolism,

453 and they were within the range of calves at this age (Knowles et al., 2000). Similarly,

454 serum urea and creatinine are indicative of protein catabolism, and kidney damage,

455 respectively, and their values were similar in all treatments. Hepatic enzyme AST is an

456 indicator of soft tissue damage and no differences were found between groups of calves.

457 Although differences appeared in the hepatic enzyme GGT, normal values of GGT in

458 calves are around 20 U/L (Klinkon and Jezek, 2014), and values under this level are not

459 considered a health problem. Similarly, an increase in serum NEFA concentrations is an

460 indicator of body fat reserve mobilization; however, serum NEFA concentrations in the

461 present study were too low to be indicative of a negative energy balance in TW calves

462 (which had greater values than UF+UV-fed calves). Lastly, an increase of serum thyroid

463 hormone in 8L-fed calves was expected since it is a hormone related to growth and

464 basal metabolic rate, and 8L-fed calves grew more compared with 4L–fed calves.

465 To determine the preference and acceptability of UF+UV water, a preference water test

466 was performed. When data were analysed with all calves together with a t-test without

467 considering their previous experience, calves clearly showed a preference for TW (0.76

468 ± 0.297). However, the confidence limits for no preference were very wide (between

469 0.91 ± 0.30 and 0.22 ± 0.45), indicating a high variability among animals. When

19

470 analysing the effects of a previous experience on water exposure, calves that were

471 consuming TW throughout the study, afterwards did not show any preference for any of

472 the waters during the preference test (preference ratio of 0.53 ± 0.08); in contrast, calves

473 previously exposed to UF+UV had a clear preference for TW during the preference test

474 (preference ratio of 0.91 ± 0.07). This outcome demonstrated firstly, the ability of

475 calves to distinguish between the two types of water, and secondly that the UF+UV

476 water obtained in the present study, in spite of not posing any important risk for the

477 animal health, has a lower acceptability and palatability than the TW water.

4. Conclusions

479 The experiment involving intestinal primary cell cultures pointed the ultrafiltration

480 treatment of reclaimed water as necessary to prevent intestinal cell damage, apoptosis,

481 and inflammation. Wastewater treated with an ultrafiltration and ultraviolet treatment

482 seems a plausible potential option for livestock drinking. However, some

483 recommendations can be drawn from the present work when considering the use of

484 reclaimed water for livestock drinking:

485 • Multi-barrier technologies for water reclamation, such as ultrafiltration and

486 ultraviolet disinfection, achieve a desirable water physicochemical and microbiological

487 quality for livestock drinking. However, high contents of soluble salts, found in the

488 present study at concentrations around the threshold limits set up for human and

489 livestock drinking water, may reduce water palatability and acceptability and

490 consequently impair water and concentrate intakes and ultimately negatively affect

491 animal performance, but without causing noticeable health afflictions.

492 • The livestock sector is a promising candidate for the reuse of urban wastewater,

493 especially during severe drought periods. However, equilibrium between reclaimed

494 water availability, water demand, technology cost, environment sustainability, and

495 animal health and performance should be evaluated when proposing a water reuse

496 scheme for livestock.

497 • Testing for the presence of human and/or animal viral fecal indicators in the

498 wastewater used for producing reclaimed water may be relevant since it could serve for

499 tracing the origin of fecal contamination that may pose a risk for animal or/and human

500 health.

501 • Using reclaimed water in the livestock sector is challenging because it requires

502 relatively important economic investments and further studies are needed to evaluate

503 long-term effects on animal health and performance are required for its potential impact

504 on food safety.

6. References

513 Bach, A., Guasch, I., Elcoso, G., Chaucherays-Durand, F.,Castex, M., Fàbregas, F.,

514 Garcia-Fruitos, E., Arís, A., 2018. J. Dairy Sci. 101:2631-2640.

515 doi.org/10.3168/jds.2017-13212

516 Bofill-Mas, S., Rusiñol, M., Fernandez-Cassi, X., Carratalà, A., Hundesa, A., Girones

517 R., 2013. Quantification of human and animal viruses to differentiate the origin

518 of the fecal contamination present in environmental samples. Biomed Res Int.

519 192089. doi: 10.1155/2013/192089.

520   Brun-Hansen, H. C., Kampen, A. H., Lund, A., 2006. Hematologic values in calves

521   during the first 6 months of life. Vet. Clin. Pathol. 35, 182-187.

522   Bruno, D. R., Rossitto,P. V., Bruno, R. G. S., Blanchard, M. T, Sitt, T., Yeargan, B. V.,

523   Smith, W. L., Cullor, J. S., Stott, J. L., 2010. Differential levels of mRNA

524   transcripts encoding immunologic mediators in mammary gland secretions from

525   dairy cows with subclinical environmental Streptococci infections. Vet.

526   Immunol. Immunopathol. 138, 15-24. doi:10.1016/j.vetimm.2010.06.009

527   Calgua, B., Fumian, T., Rusiñol, M., Rodriguez-Manzano, J., Mbayed, V. A., Bofill-

528   Mas, s., Miagostovich, M., Girones, R., 2013. Detection and quantification of

529   classic and emerging viruses by skimmed-milk flocculation and PCR in river

530   water from two geographical areas. Water Res. 47, 2797-2810. doi:

531   10.1016/j.watres.2013.02.043.

532   Challis, D.J., Zeinstra, J.S., Anderson, M.J., 1987. Some effects of water quality on the

533   performance of high yielding cows in an arid climate. Vet. Rec. 120, 12-15.

534   Clesceri, L., Greenberg, A., Eaton, A., 1998. Standard Methods for the Examination of

535   Water and Wastewater, 20th Edition United Book Press, Baltimore.

536   Daryanto, S., Wang, L., Jacinthe, P. A., 2016. Drought effects on root and tuber

537   production: A meta-analysis. Agricultural Water Management. 176, 122-131.

538   Department for Environment Food and Rural Affairs, 2012. Sustainable water for

539   livestock-WU0132.

540   randd.defra.gov.uk/Default.aspx?Module=More&Location=None&ProjectID=1

541   7363 (accessed 6 January 2019)

542   Department of Primary Industries and Regional Development, 2017. Water quality for

543   livestock. www.agric.wa.gov.au/livestock-biosecurity/water-quality-

544   livestock?nopaging=1 (accessed 6 January 2019)

545   Department of water affairs and forestry, 1996. Agricultural use: livestock watering. In

546   Water quality guidelines, vol. 5.

547   www.dwaf.gov.za/iwqs/wq_guide/Pol_saWQguideFRESHLivestockwateringvol

548   5.pdf (accessed 6 January 2019)

549   Drastig, K., Prochnow, A., Kraatz, S., Klauss, H., Plöchl, M., 2010. Water footprint

550   analysis for the assessment of milk production in Brandenburg (Germany). Adv.

551   Geosci. 27, 65-70. doi: 10.5194/adgeo-27-65-2010.

552   EPA Victoria, 2003. Guidelines for Environmental Management. Use of reclaimed

553   water. ISBN 0730676226.

554   FAO, 2015. Towards a water and food secure future. www.fao.org/3/a-i4560e.pdf

555   (accessed 6 January 2019)

556   Feldlite, M., Juanicó, M., Karplus, I., Milstein, A., 2008. Towards a safe standard for

557   heavy metals in reclaimed water used for fish aquaculture. Aquaculture 284 (1-

558   4), 115-126. doi.org/10.1016/j.aquaculture.2008.07.036

559   Gulbenkian Think Tank on Water and the Future of Humanity, 2014. Drivers of water

560   demand, course changes, and outcomes. In: Water and the Future of Humanity.

561   Springer, Cham.

562   Guy, R. A., Payment, P., Krull, U. J., Horgen, P. A., 2003. Real-time PCR for

563   quantification of Giardia and Cryptosporidium in the environmental water

564   samples and sewage. Appl. Envrion. Microbiol. 69 (9), 5178-5185. doi:

565   10.1128/AEM.69.9.5178–5185.2003

566   Hernroth, B. E., Conden-Hansson, A. C., Rehnstam-Holm, A. S., Girones, R., Allard,

567   A. K., 2002. Environmental factors influencing human viral pathogens and their

568   potential indicator organisms in the blue mussel, Mytilus edulis: the first

569   Scandinavian report. Appl. Environ. Microbiol. 68, 4523-4533. Appl. Envrion.

570   Microbiol. 72 (12), 7894-7896. doi: 10.1128/AEM.68.9.4523–4533.2002

571 House, C. H., Bergmann, B. A., Stomp, A. M., Frederick, D. J., 1999. Combining

572   constructed wetlands and aquatic and soil filters for reclamation and reuse of

573   water. Ecol. Eng. 12 (1-2), 27-38. doi.org/10.1016/S0925-8574(98)00052-4

574 Hundesa, A., S. Bofill-Mas, C. Maluquer de Motes, J. Rodriguez-Manzano, A. Bach,

575   M. Casas, and R. Girones. 2010. Development of a quantitative PCR assay for

576   the quantitation of bovine polyomavirus as a microbial source-tracking tool. J.

577   Virol. Methods. 163:385–389. doi: 10.1016/j.jviromet.2009.10.029.

578 Jiang, Y., xu, X., Huang, Q., Huo, Z., Huang, G., 2016. Optimizing regional irrigation

579   water use by integrating a two-level optimization model and an agro-

580   hydrological model. Agric. Water Manag. 178, 76-88.

581   doi.org/10.1016/j.agwat.2016.08.035

582 Jjemba, P. K., Weinrich, L. A., Cheng, W., Giraldo, E., LeChevallier, M. K., 2010.

583   Regrowth of potential opportunistic pathogens and algae in reclaimed-water

584   distribution systems. Appl. Environ. Microbiol. 76 (13), 4169-4178. doi:

585   10.1128/AEM.03147-09

586 Kertz, A. F., Reutzel, L. F., Mahoney, J. H., 1984. Ad libitum water intake by neonatal

587   calves and its relationship to calf starter intake, weight gain, feces score, and

588   season. J. Dairy Sci., 67, 2964-2969.

589 Kihila, J., Mtei, K. M., Njau, K. N., 2014. Wastewater treatment for reuse in urban

590   agriculture; the case of Moshi Municipality, Tanzania. Physics and Chemistry of

591   the Earth, Parts A/B/C. 72-75, 104-110.

592 Klinkon, M., Jezek, J., 2014. Values of blood variables in calves. A bird's-eye view of

593   veterinary medicine. Dr. Carlos C. Perez-Marin (Ed.), ISBN: 978-953-51-0031,

594   InTech, available from: www.intechopen.com/

595 Knowles, T. G., Edwards, J. E., Bazeley, K. J., Brown, S. N., Butterworth, A., Warriss,

596   P. D., 2000. Changes in blood biochemical and haematological profile of

597   neonatal calves with age. Vet. Rec. 147, 593-598.

598 Kusabe, A., Contreras-Barragan, B. A., Simpson, C. R., Enciso, J. M., Nelson, S. D.,

599   Melgar, J. C., 2016. Application of partial rootzone drying to improve irrigation

600   water use efficiency in grapefruit trees. Agric. Water Manag. 178, 66-75.

601   doi.org/10.1016/j.agwat.2016.09.012

602 Li, D., Zeng, S., Gu, A. Z., He, M., Shi, H., 2013. Inactivation, reactivation and

603   regrowth of indigenous bacteria in reclaimed water after chlorine disinfection of

604   a municipal wastewater treatment plant. J. Envrion. Sci. 25 (7), 1319-1325.

605 Maestre-Valero, J. F., Martin-Gorriz, B., Alarcón, J. J., Nicolás, E., Martinez-alvarez,

606   V., 2016. Economic feasibility of implementing regulated deficit irrigation with

607   reclaimed water in a grapefruit orchard. Agric. Water Manag. 178, 119-125.

608   doi.org/10.1016/j.agwat.2016.09.019

609 Mekonnen, M. M., Hoekstra, A. Y., 2012. A global assessment of the water footprint of

610   farm animal products. Ecosystems 15, 401-415. doi: 10.1007/s10021-011-9517-

611   8

612 Nishimura, R., Komiyama, J., Tasaki, Y., Acosta, T. J., Okuda, K., 2008. Hypoxia

613   promotes luteal cell death in bovine corpus luteum. Biol. Reprod. 78, 529-536.

614   doi 10.1095/biolreprod.107.063370

615 Olkowski, A. A., 2009. Livestock water quality: a field guide for cattle, horses, poultry

616   and swine. Ministry of Agriculture and Agri-Food Canada.

617 Pfaf, M.W., 2014. Quantification strategies in real-time PCR. In S. A. Bustin, A-Z of

618   quantitative PCR (pp. 87–112). International University Line, La Jolla, CA

619 Pina, S., Puig, M., Lucena, F., Jofre, J., Girones, R., 1998. Viral pollution in the

620   environment and in shellfish: human adenoirus detection by PCR as an index of

621   human viruses. Appl. Environ. Microbiol. 64, 3376-3382.

622 Ran, Y., Lannerstad, M., Herrero, M., Van Middelaar, C. E., De Boer, I. J. M., 2016.

623   Assessing water resource use in livestock production: a review of methods.

624   Livest. Sci. 187, 68-79. doi.org/10.1016/j.livsci.2016.02.012

625 RD 140/2003. Royal Decree establishing the sanitary criteria for the quality of drinking

626   water (Real Decreto por el que se establecen los criterios sanitarios de la calidad

627   del agua de consumo). BOE num. 45, 7228-7245.

628 Richardson, S. D., Plewa, M. J., Wagner, E. D., Schoeny, R., DeMarini, D. M., 2007.

629   Occurrence, genotoxicity, and carcinogenicity of regualted and emerging

630   didsinfection by-products in drinking water: A review and roadmap for research.

631   Mutat. Res. 636 (1-3), 178-242.

632 Riollet, C., Rainard, P., Poutrel, B., 2001. Cell subpopulations and cytokine expression

633   in cow milk in response to chronic *Staphylococcus aureus* infection. J. Dairy

634   Sci. 84:1077-1084.

635 Roland, L., Drillich, M., Iwersen, M., 2014. Hematology as a diagnostic tool in bovine

636   medicine. J. Vet. Diagnos. Invest. 26, 592-598.

637 Schlink, A. C., Nguyen, M. L., Viljoen, G. J., 2010. Water requirements for livestock

638   production: a global perspective. Rev. Sci. Tech. Off. Int. Epiz. 29 (3), 603-619.

639 Shannon, M. A., Bohn, P. W., Elimelech, M., Georgiadis, J. G., Mariñas, B. J., Mayes,

640   A. M., 2008. Science and technology for water purification in the coming

641   decades. Nature 452 (7185), 301-310. doi: 10.1038/nature06599.

642 Singh, S., Angadi, S., Grover, K. K., St. Hilaire, R., Begna, S., 2016. Effect of growth

643   stage based irrigation on soil water extraction and water use efficiency of spring

644   safflower   cultivars.   Agric.   Water   Manag.   177,   432-439.

645   doi.org/10.1016/j.agwat.2016.08.023

646   Smol., M., Wladarczyk, M., 2012. Effectiveness in the removal of polycyclic aromatic

647   hydrocarbons from industrial wastewater by ultrafiltration technique. Arch.

648   Environ. Protect. 38 (4) 49-58. doi: 10.2478/v10265-012-0040-6

649   Solomon, R., Miron, J., Ben-Ghedalia, D., Zomberg, Z., 1995. Performance of high

650   producing dairy cows offered drinking water of high and low salinity in the

651   Arava desert. J. Dairy Sci. 78, 620-624.

652   Terré, M., Tejero, C., Bach, A., 2009. Long-term effects on heifer performance of an

653   enhanced-growth feeding programme applied during the preweaning period. J.

654   Dairy Res. 76, 331-339. doi: 10.1017/S0022029909004142

655   Vurukonda. S. S. K. P., Vardharajula, S., Shrivastava, M., SkZ, A., 2016. Enhancement

656   of drought stress tolerance in crops by plant growth promoting rhizobacteria.

657   Microbiol. Res. 184, 13-24. doi: 10.1016/j.micres.2015.12.003.

658   Yuan, J., Liu, H., Zhou, L. H., Lu, W.Q., 2006. Oxidative stress and DNA damage

659   induced by drinking-water chlorination byproduct 3-chloro-4-(dichloromethyl)-

660   5-hydroxy-2(5H)-furanone (MX) in mice. Mutat Res. 609, 129-136.

661

1   **Figure 1.** Expression of apoptotic (a), cell damage (b) and inflammation (c) genes,

2   expressed as relative media culture sample folds, of *in vitro* intestinal cells cultured with

3   different types of water: cells media culture (MC), tap water (TW), dairy cow trough

4   water that was filled with chlorinated ground water (ThW), secondary effluent from

5   Caldes WWTP (SW), secondary effluent with an ultrafiltration (UF), secondary effluent

6   with an UV disinfection (UV), secondary effluent with UF and UV treatments

7   (UF+UV), and secondary effluent with UF and chlorination (UF+ClO$^-$). Columns with

8   different letters indicate differences within gene among water treatments.

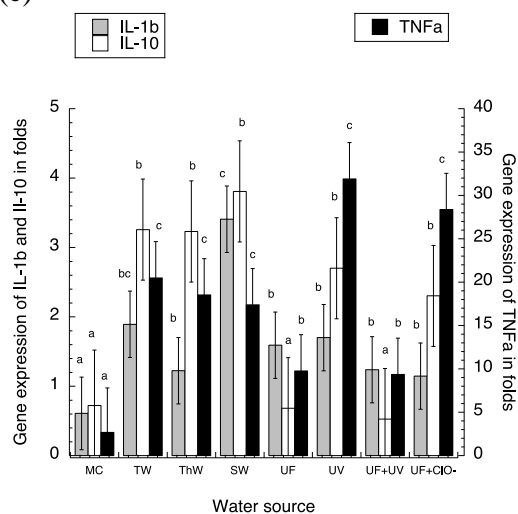9   (a)   (b)
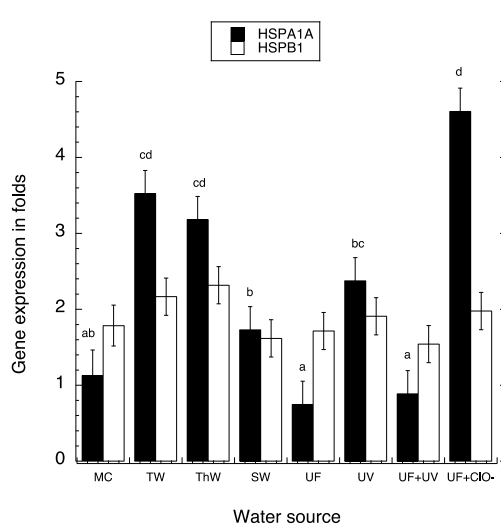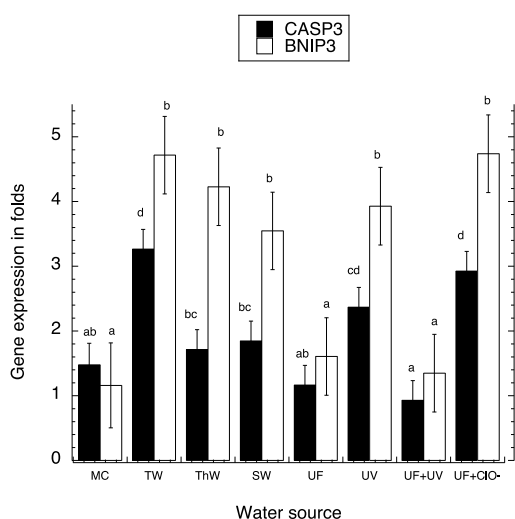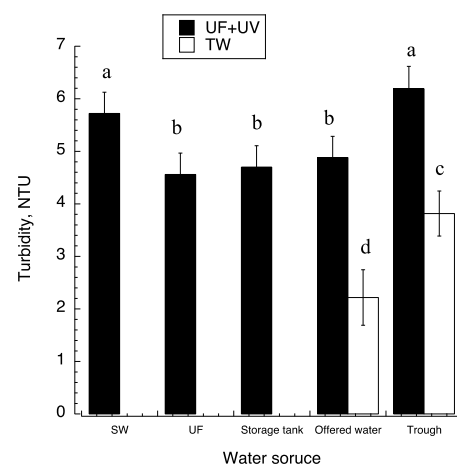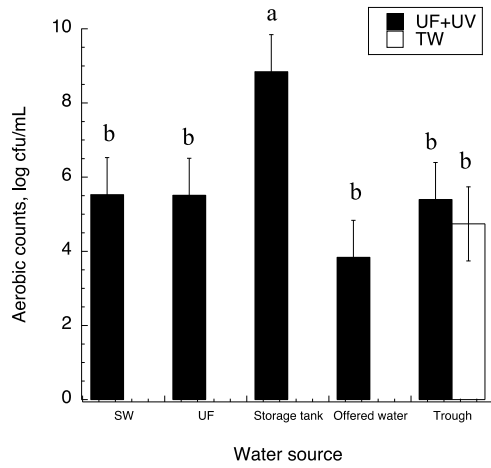

10

11   (c)

12

1   **Figure 2.** Evolution of aerobic counts (a) and turbidity (b) of UF+UV reclaimed water

2   during the production process in the discontinuous system, and the final quality in

3   animal troughs.

4   (a)   (b)

5

6

7

8

Table 1. Water chemical and microbiological quality parameters of the different waters used in the *in vitro* Study 1.

	Source of water[1]						
	TW	ThW	SW	UF	UV	UF+UV	UF+ClO⁻
pH	7.8	7.8	7.5	7.3	7.5	7.5	7.5
Conductivity, µS/cm	570	601	980	943	985	971	951
Turbidity, NTU	0.6	3.7	2.5	0.6	2.3	1.1	1.3
COD, mgO_2/L	< 50	< 50	< 50	< 50	< 50	< 50	< 50
NH_4^+, mg/L	< 0.1	< 0.5	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
Chloride, mg/L	36	41.5	173	165	173	174	167
Phosphate, mg/L	< 0.2	< 0.5	1.5	1.6	1.2	1.2	1.2
Nitrate, mg/L	9.9	< 0.5	19	18	17	17	17
Sulphate, mg/L	45	ND	45	42	43	43	42
Aerobic counts, cfu/mL	0	1,540,000	205,000	< 150	4	1	0
E. coli, cfu/mL	< 5	58	7,600	< 5	< 5	< 5	< 5

[1] TW= tap water; ThW= water from an animal trough; SW= secondary effluent of a wastewater treatment plant; UF= secondary effluent with an ultrafiltration treatment; UV= secondary effluent with an ultraviolet disinfection; UF+UV= secondary effluent with an ultrafiltration treatment and an ultraviolet disinfection; UF+ClO⁻= secondary effluent with an ultrafiltration treatment and a chlorination

ND = not determined

Table 2. Gene names, primer sequences, annealing temperature, primer concentration, and efficiency of the used genes in Study 1.

Gene name	Primer sequence (5' to 3')		Tm	µM	Efficiency	Reference
β-actin (*ACTB*)	Fw	CTGGACTTCGAGCAGGAGAT	57ºC	0.125	1.82	Bach et al., 2018
	Rv	CCCGTCAGGAAGCTCGTAG				
Tumor necrosis factor alpha (TNF-α)	Fw	AACAGCCCTCTGGTTCAAAC	60ºC	0.5	1.89	Riollet et al., 2001
	Rv	TCTTGATGGCAGACAGGATG				
Interleukin 1 beta (IL-1ß)	Fw	TGGGAGATGGAAACATCCAG	50ºC	0.3125	1.82	Riollet et al., 2001
	Rv	TTTATTGACTGCACGGGTGC				
Interleukin 10 (IL-10)	Fw	ACTTTAAGGGGTTACCTGGGTTG	57ºC	0.5	1.90	Bruno et al., 2010
	Rv	GAAAGCGATGACAGCGCCGC				
Adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3)	Fw	GAAGGAATGCCGACACTAGG	55ºC	0.5	1.85	Nishimura et al., 2008
	Rv	CAAAGCCAGCAGACACTCAG				
Caspase 3 (CASP3)	Fw	AAGCCATGGTGAAGAAGGAA	55ºC	0.5	1.88	Nishimura et al., 2008
	Rv	GGCAGGCCTGAATAATGAAA				
Heat shock 70 kDa protein 1A (HSPA1A)	Fw	GGCACCAGAGCTTCACGATGT	60ºC	0.5	1.91	Bach et al., 2018
	Rv	CCTACGCAGGAGTAGGTGGT				
Heat shock protein family B member 1 (HSPB1)	Fw	CCTGAAACACCGCCTGCTAA	60ºC	0.5	1.92	Bach et al., 2018
	Rv	CGGAGAAGCGAGAGAAGTGG				

Table 3. Physicochemical and microbiological analysis of tap water (TW) and ultrafiltered and ultraviolet treated wastewater (UF+UV) used in Study 2 to prepare milk replacer and as drinking water for young calves.

	Type of water				Upper limits
	TW	UF+UV	SEM	P-value	(Schlink et al., 2010)
pH	7.8	8.4	0.07	0.001	-
Conductivity, μS/cm	570	1,437	38.8	<0.001	-
Total suspended solids, mg/L	1.33	0.92	0.236	0.29	-
Turbidity, NTU	0.6	1.8	1.23	0.53	-
Total organic carbon, mg/L	2.0	4.9	0.43	<0.01	-
Total inorganic carbon, mg/L	40.0	46.9	-	-	-
Total dissolved solids, mg/L	384	756	-	-	-
Chloride, mg/L	36	269	5.03	<0.01	100
Sulphate, mg/L	45.3	56.7	4.11	0.19	50
Bromide, mg/L	0.55	0.45	0.403	0.88	-
Nitrate, mg/L	9.9	16.4	1.05	0.05	89
Nitrite, mg/L	< 0.2	< 0.2	-	-	-
Phosphate, mg/L	< 0.2	4.5	0.57	0.03	2.15
Ca^{2+}, mg/L	57.8	74.8	4.75	0.13	100
Mg^{2+}, mg/L	18.5	19.5	2.68	0.82	50
Na^+, mg/L	20.3	188.1	1.34	<0.001	50
K^+, mg/L	2.8	20.2	0.11	<0.001	20
NH_4^+, mg/L	< 0.2	< 0.2	0.03	0.67	-
Total Mn, μg/L	2.3	11	-	-	50

Total Fe, µg/L	5.7	15	-	-	200
Aerobic counts, cfu/mL	ND[1]	1.4E+05	2.04E+05	-	-
Total coliforms, cfu/mL	0	1.6	-	-	-
Escherichia coli, cfu/mL	0	0.4	0.9	-	-
Clostridium perfringens, cfu/mL	0	0	-	-	-
Cryptosporidium, spp, oocyst/L	ND[1]	< 8	-	-	-
Bovine polyomaviruses, GC/L	ND[1]	< 105	-	-	-
Human adenoviruses, GC/L	ND[1]	< 105	-	-	-
Somatic coliphages, pfu/mL	ND[1]	5	5.8	-	-
Helminth eggs, egg/10L	ND[1]	< 1	-	-	-
Taenia spp eggs, egg/10 L	ND[1]	< 1	-	-	-

[1] Not determined

1 **Table 4.** Performance and dry matter (DM) intake of calves fed two different milk

2 feeding programs (4 L/d *vs* 8 L/d of milk replacer), with two different sources of water:

3 tap water (TW) *vs* ultrafiltered and ultraviolet (UF+UV) treated wastewater.

	Treatments					P-values[1]		
Water source	TW		UF+UV					
Milk program	4L	8L	4L	8L	SEM[2]	W	M	WxM
Number calves	4	4	5	5	-	-	-	-
Initial age, d	5.3	3.5	4.8	5.4	1.63	0.66	0.73	0.48
Initial body weight, kg	40.6	37.8	40.0	41.0	3.21	0.69	0.79	0.57
Final body weight, kg	66.0	71.9	63.3	69.3	1.57	0.35	0.001	0.60
Average daily gain, g/d	616	778	556	692	49.1	0.14	0.003	0.79
Dry matter intake, g/d								
Milk replacer	487	861	487	872	0.02	0.72	<0.001	0.73
Concentrate	650	282	532	170	45.6	0.01	<0.001	0.94
Straw	17	15	14	15	7.6	0.81	0.94	0.85
Water intake, L/d	3.4	3.2	2.9	2.8	0.41	0.19	0.64	0.86
Feed efficiency[3]	0.53	0.68	0.53	0.69	0.04	0.89	<0.001	0.94

4 [1] W: effect of the type of water used to prepare the MR and for drinking calves; M:
5 effect of the volume of MR offered to calves; WxM: effect of the interaction of source
6 of water and amount of milk replacer
7 [2] standard error of the mean
8 [3] Expressed as ratio between daily gain and daily feed consumption
9