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Title:

Increase of *Clostridium perfringens* in association with *Eimeria* in haemorrhagic enteritis in Japanese beef cattle

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Abstract

A coprological survey with detailed clinical observation of naturally occurring haemorrhagic enteritis (HE) cases was conducted to understand the pathophysiology of HE by clarifying the infection status of *Eimeria* and enteropathogenic bacteria in cattle. Faecal samples from 55 cases of HE and 26 clinically normal animals were collected and a quantitative examination of *Eimeria* and potential enteropathogenic bacteria was performed. The number of *Eimeria* spp. oocysts per gram of faeces (OPG) exceeded 10,000 in 69.1 per cent of HE cases with a maximum of 1,452,500 OPG and *E. zuernii* was found to be overwhelmingly dominant. A significant increase in faecal coliform count was observed in HE cases when compared to clinically normal animals. Among the animals shedding more than 10,000 OPG, 42.9 per cent showed a remarkable increase in C*lostridium perfringens* abundance ($>10^4$ CFU/g) in the faeces. In the cases with *C. perfringens* detected, its abundance was positively correlated with *Eimeria* OPG and high *C. perfringens* abundance was always accompanied by high *Eimeria* OPG. *E. zuernii* is likely to play a crucial role in massive multiplication of *C. perfringens* in HE in cattle.

Introduction

Bovine coccidiosis, often caused by *Eimeria bovis* and *Eimeria zuernii* infections, is regarded as one of the most important diarrhoeal diseases in cattle, causing moderate diarrhoea to severe life-threatening haemorrhagic enteritis (Friend and Stockdale 1980, Stockdale and others 1981). These protozoa are found worldwide and the vast majority of cattle are exposed to them at some time in their lives. Calves between three weeks to six months old are particularly susceptible to the infection resulting in clinical coccidiosis (Oda and Nishida 1990, Taylor and Catchpole 1994, Daugschies and Najdrowski 2005, Matsubayashi and others 2009). This has led to a number of reports and studies describing the clinical significance and treatments for the disease in calves (Stockdale and others 1981, Bangoura and others 2007, Jonsson and others 2011). Cases of severe coccidiosis with bloody diarrhoea in mature animals have also often been observed in fattening farms, especially in Japanese Black (full-blood Wagyu) as reported by various Prefectural Federation of Agricultural Relief Associations and Prefectural Livestock Hygiene Centres in Japan. Unfortunately, few documents concerning the aetiology have been published internationally (Sato and others 2010). For these cases, conventional treatment methods blindly relying on the use of anticoccidial drugs often did not produce satisfactory results. Further investigation of the aetiological

characterization of the disease in fattening cattle, potentially also taking into consideration the effect of co-infection with other pathogens, is required (Kano and others 2011).

It has been demonstrated in pigs (Mengel and others 2012) and chickens (Collier and others 2008) that coccidial infections can lead to severe enteritis associated with *Clostridium perfringens*. However, such studies are virtually lacking for bovine coccidiosis even though it is conceivable that *Eimeria* infection in cattle may also play a crucial role in the colonization and/or proliferation of other pathogens such as enteropathogenic bacteria. This study explores the infection status of *Eimeria* and potential enteropathogenic bacteria in haemorrhagic enteritis cases observed in Japanese beef cattle including calves and fattening cattle to understand the pathophysiology of this disease.

Materials and methods

Sample collection

The samples were collected between April and July 2012 from commercial farms in Kyushu (southern island of Japan, nine farms) and Tohoku (northeast region of the main island of Japan, five farms), the university's educational farm (Livestock Science Station, University of Miyazaki) and a slaughterhouse in South Kyushu.

Faecal samples were collected from a haemorrhagic enteritis (HE) group and a control group. During the study period, all cases suffering from significant bloody diarrhoea in the targeted regions in Kyushu and Tohoku, regardless of their sex or age, were included in the HE group for a total of 55 animals. The sample size was determined by expected population variance and statistical power. Faecal samples were collected on the first day of illness before any treatments or therapies. Faecal samples for the control group were taken from 15 apparently healthy animals in a slaughterhouse and 11 from the Livestock Science Station, University of Miyazaki, Miyazaki, Japan. Breed, age, sex and clinical findings were recorded for each animal.

Clinical observations

Faecal consistency and the extent of bleeding in the HE group were immediately assessed at sample collection and classified using a faecal score key from 1 to 3, as shown in Table 1.

As well as faecal scores, animals underwent qualitative evaluations of clinical scores by observing eight clinical signs of enteritis: fever, hypothermia, anorexia, dehydration, expression of celialgia, straining, excretion of pseudomembrane and/or tissue and abdominal water sounds. Clinical scores were determined as cumulative scores (clinical score 0 to 8) by counting the number of the above clinical signs observed in each animal.

Parasitological examination

Faecal samples collected from both HE and control groups were tested for oocysts and helminth eggs. The number of oocysts and helminth eggs per gram of faeces (OPG and EPG, respectively) were determined by the modified McMaster's method (Thienpont and others 1986), with a sensitivity of 100 OPG/EPG, using a saturated salt solution with a specific gravity of 1.2. Identification of *Eimeria* species (*E. alabamensis*, *E. auburnensis*, *E. bovis*, *E. ellipsoidalis*, *E. zuernii* and others) was based on the morphology described (Levine and Ivens 1967, Levine 1985).

Determination of faecal bacteria abundance

Faecal samples from all animals were examined microbiologically for *Clostridium perfringens*, *Salmonella* spp., and coliforms by cultivation in each specific culture media: 1) *Clostridium perfringens*, CW Agar with kanamycin (KCW) "Nissui" with egg yolk (10 per cent v/v) under anaerobic conditions; 2) *Salmonella* spp., Selenite Cystine Broth "Nissui" with Sodium Selenite (0.4 per cent w/v) and EEM Broth "Nissui" (all above, Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) for preculture followed by selective culture on ES Salmonellae Agar II "Eiken" (Eiken Kagaku, Tokyo, Japan) or CHROMagar TM Salmonella (CHROMagar Microbiology, Paris, France); 3) coliforms, Deoxycholate Hydrogen Sulfide Lactose (DHL) Agar "Nissui" (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan). The bacterial abundance was examined using the plate colony count method.

Serum vitamin A

To observe the degree of vitamin A deficiency, serum samples from the HE group were collected and stored at -20°C until analysed. The concentration of vitamin A in serum was measured by HPLC (Suhara and Kanei 1992). Serum vitamin A levels in the HE group were compared with reference data from mass profile tests conducted for farm consultations in the study area.

Statistics

Associations between variables were analysed using the Spearman's rank correlation or Wilcoxon rank-sum test. The Steel-Dwass test was used for multiple comparisons. A nominal significance level of 5 per cent (α=0.05) was used for all statistical tests. All analyses were performed using Statistical program R (http://www.r-project.org). The results are expressed as the mean value with standard error of the mean (SEM), unless otherwise indicated.

Ethical Statement

All faecal and blood sampling protocols were performed with appropriate veterinary care and involved oral informed consent from the owners of the animals in the study.

Results

Haemorrhagic enteritis cases

The HE group consisted of 47 Japanese Black (full-blood Wagyu) and eight F1 crossbred (Japanese Black × Holstein) beef cattle. Animals were aged between two and 48 months and were 27 fattening cows, 20 fattening steers, seven calves (three female, four male) and a beef dam.

When the fattening cows and steers in the HE group were classified by age group at three-month intervals to see the age distribution, they distributed intensively at nine to 11 months old (19.1 per cent), 18 to 20 months old (21.3 per cent), 21 to 23 months old (17.0 per cent), and 24 to 26 months old (23.4 per cent) (Fig. 1).

Differences in the number of cases, faecal and clinical scores, oocyst and germ counts, or serum vitamin A level were not observed between sexes.

Eimeria OPG

The arithmetic mean OPG of *Eimeria* spp. in the HE and control groups was 193,390±41,509 and 58±43, respectively, showing a significant difference between them ($P<0.001$) (Fig. 2A). Cases shedding more than 10,000 oocysts in a gram of faeces reached 69.1 per cent in the HE group (38/55), with a maximum

OPG of 1,452,500. OPGs of three samples (5.5 per cent) in the HE group and 22 samples in the control group (87.6 per cent) were below the limit of detection. The HE group was classified into sub-groups according to their OPG classes, and the mean proportion of specific OPG for each *Eimeria* species in the total OPG was calculated (Fig. 3). *E. zuernii* was observed as an overwhelmingly dominant species in 83.6 per cent of the samples from the HE group. When the total OPG was 1000 to 9900, the mean percentage of *E. zuernii* was 80.8±8.8 per cent. However, when the total OPG was 10,000 to 99,900, 100,000 to 999,900, or above 1,000,000 the mean percentages rose to 94.0±1.7 per cent, 95.9±1.1 per cent and 97.1±1.8 per cent respectively. In samples with OPGs less than 1000, the dominant species was *E. bovis* followed by *E. auburnensis*. The higher the OPG, the higher the proportion of *E. zuernii* (rs=0.52, $P<0.001$).

Bacterial counts

The first 30 samples collected in the HE group were qualitatively examined for all of the targeted bacteria to give an overview of the bacterial burden in these clinical cases. Coliform bacteria were detected from all samples and *C. perfringens* was detected in 24 samples. Quantification of coliforms was then done on 25 samples in the HE group and 16 samples in the control group. Among them, 19 samples in the HE group and 16 samples in the control group were submitted

202 for quantification of *C. perfringens*. The mean faecal coliform count (CFU/g) was
203 significantly higher in the HE group ($5.6\times10^9\pm2.7\times10^9$) than the control group
204 ($8.7\times10^5\pm4.6\times10^5$) ($P<0.001$) (Fig. 2B).
205 The difference in the mean faecal *C. perfringens* counts (CFU/g) was not
206 significant between the HE group ($2.3\times10^7\pm1.5\times10^7$) and the control group
207 ($3.9\times10^2\pm1.4\times10^2$) (Fig. 2C). However, when analysis was performed on samples
208 (n=21) showing detectable *C. perfringens*, a remarkable increase in *C. perfringens*
209 ($>10^4$ CFU/g) was observed only in the HE group and the increased bacterial count
210 for *C. perfringens* was significantly correlated with increased *Eimeria* spp. OPG
211 ($rs=0.55$, $P<0.001$) (Fig. 4).
212 *Salmonella* spp. were not detected in any sample in either group.
213
214 **Faecal and clinical scores**
215 HE group faeces presented various levels of haemorrhagic appearance
216 from a reddish brown paste to a red wine-like liquid (Fig. 5). Twenty-one samples
217 (38.2 per cent) were classified as “faecal score 1”, 18 (32.7 per cent) as “faecal
218 score 2”, and the remaining 16 (29.1 per cent) were marked “faecal score 3”.
219 Clinical signs observed at the onset of illness were mild in most cases in
220 the HE group. The number of cases classified into “clinical score 0” “clinical score
221 1”, “clinical score 2” and “clinical score 3” was 25 (45.5 per cent), 12 (21.8 per

222 cent), nine (16.4 per cent) and four (7.3 per cent), respectively, and maximum
223 clinical scores of 4 were recorded in five cases (9.1 per cent). The most frequent
224 clinical sign was anorexia (24/55) followed by fever (13/55), straining (8/55),
225 hypothermia (5/55) and excretion of pseudomembrane and/or tissue (5/55),
226 dehydration (3/55), expression of celialgia (2/55) and abdominal water sounds
227 (2/55).

228 Faecal score values significantly increased relative to the *Eimeria* OPG
229 (P=0.014, rs=0.3). Mean OPG was higher when faecal scores were either 2 or 3
230 compared to those for faecal score 1 (Fig. 6).

231

232 **Serum vitamin A**

233 Blood samples were collected from all animals in the HE group except
234 for four heads of fattening cattle, seven calves and a beef dam. For the analysis of
235 serum vitamin A levels, the values of two animals were excluded from the data
236 because they had been given vitamin A supplement as part of a regular maintenance
237 a few days before blood collection.

238 The remaining 41 animals showed serum vitamin A concentrations
239 ranging from 4.3 to 80.0 IU/dL with a mean of 33.3±2.9 IU/dL. When compared
240 with the age-associated curve in the change of serum vitamin A levels in normal
241 fattening cattle, 14 animals (35.0 per cent) in the HE group showed lower values

242 (Fig. 7).

243

Discussion

An increased rate of excretion of *Eimeria* oocysts was observed in clinical cases of HE in this study. The majority of total OPGs in HE cases were represented by a single species, *E. zuernii*, which is the most pathogenic of the 13 bovine *Eimeria* species observed in Japan. On the other hand, the *Eimeria* OPG in the control group was extremely low or below the detection limit and was assumed to be at least partly because of protective immunity to subsequent homologous infections (Sühwold and others 2010) in conventional conditions where animals are unavoidably exposed to *Eimeria* oocysts (Daugschies and Najdrowski 2005). The animals shedding millions of *E. zuernii* oocysts in the HE group may have failed to acquire immunity to protect themselves against a massive infection of the species (or the strain) over the rearing period. However, the factors that determine the predominance of a certain species in mixed infections are still unclear.

Conversely, there were three animals in the HE group that did not shed detectable amounts of oocysts despite the obvious presence of bloody diarrhoea. Their ages were two, 22 and 29 months and their coliform counts ranged from 10^6 to 10^7. Although it can be considered that the intestinal damage might have been caused by other pathogens, for example, coronavirus or rotavirus (Dea and others 1995), rather than *Eimeria*, it is also likely that the diarrhoeic faeces and large

amounts of blood, tissue or mucus shed in faeces interfered with the aetiological diagnosis by coproscopy in these cases. The sensitivity of coproscopical methods is particularly reduced in severe *E. bovis* or *E. zuernii* infections because oocysts are further diluted in diarrhoeic faeces or trapped within tissue and fibrin shed in bloody diarrhoea (Daugschies and Najdrowski 2005). It is also possible that clinical signs develop before oocyst shedding starts (Daugschies and Najdrowski 2005, Mundt and others 2005).

The faecal coliform count in the HE group showed more than a 6000-fold increase compared to that of the control group (Fig. 2B). This indicates that a dynamic change in the intestinal microbial ecosystem occurs in the early stage of disease, which may allow uncontrolled proliferation of particular organisms.

Co-infection with coccidia and *C. perfringens* in necrotic enteritis has been well documented in poultry and piglets (McDougald 1998, Westphal and others 2007). Type A strains of *C. perfringens* cause diarrhoea, dysentery and enterotoxaemia in ruminants, pigs, horses and poultry (Lebrun and others 2010). Information on the mechanism of *C. perfringens*–induced necrotic enteritis associated with *Eimeria* infection in poultry has recently emerged (Collier and others 2008, Wu and others 2014), but it is still far less clear in mammals. A great abundance of *C. perfringens* was observed in the HE group with a mean of 2.3×10^7 CPU/g. In cattle, counts higher than 10^6 to 10^7 CFU/ml of intestinal contents are

284 considered as a hallmark of *C. perfringens* overgrowth and confirmatory of field
285 cases (Lebrun and others 2010). Although *C. perfringens* counts did not show any
286 correlation with clinical outcome in the HE group, it was positively correlated to
287 *Eimeria* OPG in the cases with detectable *C. perfringens*. Among the faecal
288 samples in this study, high *C. perfringens* counts were always accompanied by high
289 *Eimeria* spp. OPG with exclusive dominancy of *E. zuernii*. The samples with high
290 *C. perfringens* count (>10^4 CFU/g) but low oocyst counts were only 3.3 per cent
291 while those with high OPG (>10,000) but low *C. perfringens* count (<10^4 CFU/g)
292 reached 26.7 per cent of all samples examined (Fig. 4). This supports the
293 assumption that *E. zuernii* paves the way for extensive development of *C.*
294 *perfringens* infection rather than *vice versa*.

295 Haemorrhagic enteritis cases were observed with a higher frequency in
296 animals at nine to 11 and 18 to 26 month of age (Fig. 1).

297 Japanese beef cattle producers normally sell their calves at nine to 10
298 months of age. Then after long distance transportation from calf-rearing farms to
299 fattening farms, calves are immediately grouped with other calves from different
300 farms and are suddenly exposed to a variety of infectious pathogens. The stress
301 response to transportation and regrouping results in immune suppression and an
302 increase in pathogen shedding (Swanson and Morrow-Tesch 2001, Veissier and
303 Boissy 2001, Gupta and Earley 2005). These conditions could explain the higher

304 frequency of haemorrhagic enteritis cases observed in animals at nine to 11 months
305 of age in this study.

306 On the other hand, it is common practice during the fattening stage of
307 Japanese beef cattle to reduce serum vitamin A levels by giving vitamin A-deficient
308 feed to increase the marbling in meat (Oka and others 1998, Nade and others 2003).
309 The majority of the HE group showed vitamin A levels within the reference range
310 and the correlation between vitamin A level and clinical score was weak (rs=0.35,
311 P=0.022). However, as shown in Figs. 1 and 7, the HE cases were observed
312 intensively in age groups with low serum vitamin A levels in the middle to the
313 second half of fattening process. This can be explained by the influence of the
314 vitamin A dietary metabolite retinoic acid on intestinal homeostasis (Maloy and
315 Powrie 2011). Thus serum vitamin A deficiency is also likely to be one of the risk
316 factors of haemorrhagic enteritis in beef cattle.

317 Based on data obtained in this study, *E. zuernii* is likely to play a crucial
318 role in massive multiplication of *C. perfringens* under certain conditions, such as
319 stress in response to transportation or vitamin A deficiency, which underpins the
320 pathophysiology demonstrated in pigs (Mengel and others 2012) and chickens
321 (Collier and others 2008). Both *E. zuernii* and clostridia are widely distributed in
322 cattle farms, and thus, severe *E. zuernii* infection and subsequent overgrowth of *C.*
323 *perfringen*s in the intestine appear to be a likely event in conventional herds. Taken

together, conventional HE treatment practices should be reconsidered paying special attention to the influence of *C. perfringens* and its enterotoxin on the pathophysiology. More importantly, *Eimeria* control programs may therefore prevent subsequent events caused by uncontrollable multiplication of enteropathogenic bacteria including *C. perfringens*.

To our knowledge, this is one of the first coprological surveys associated with detailed clinical observations on naturally occurring coccidiosis with haemorrhagic enteritis. This disease in fattening cattle herds has been reported mainly within Japan, where a unique fattening process including nutritional manipulation is popularly employed on Japanese Black cattle. Therefore, genetic background and the environment that the cattle are raised in constitute important factors in the occurrence of the disease. Although the breed, well known as "Wagyu", has been long found exclusively in Japan, it is now spreading across the world and is being farmed intensively in many countries including Australia and United States (Elías Calles and others 2000, Polkinghorne and others 2011). The disease, therefore, needs to be brought to the attention of farmers and veterinarians not only in Japan, but also globally.

The study also indicates the importance of understanding the molecular basis of host mucogenic responses to *Eimeria* infection in mammals. Such knowledge will have profound implications for the interaction between parasites

344 and bacteria in mammalian intestinal mucosa, which is predicted to be the principal

345 pathophysiology in a wide variety of intestinal parasitoses.

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References

BANGOURA, B., DAUGSCHIES, A. and FUERLL, M. (2007) Influence of experimental Eimeria zuernii infection on clinical blood chemistry in calves. *Veterinary parasitology* **150**, 46–53.

COLLIER, C.T., HOFACRE, C.L., PAYNE, A.M., ANDERSON, D.B., KAISER, P., MACKIE, R.I. and GASKINS, H.R. (2008) Coccidia-induced mucogenesis promotes the onset of necrotic enteritis by supporting Clostridium perfringens growth. *Veterinary immunology and immunopathology* **122**, 104–15.

DAUGSCHIES, A. and NAJDROWSKI, M. (2005) Eimeriosis in cattle: current understanding. *Journal of veterinary medicine. B, Infectious diseases and veterinary public health* **52**, 417–27.

DEA, S., MICHAUD, L. and MILANE, G. (1995) Comparison of bovine coronavirus isolates associated with neonatal calf diarrhoea and winter dysentery in adult dairy cattle in Québec. *The Journal of general virology* **76**, 1263–70.

ELÍAS CALLES, J.A., GASKINS, C.T., BUSBOOM, J.R., DUCKETT, S.K., CRONRATH, J.D., REEVES, J.J. and WRIGHT, R.W. (2000) Differences among Wagyu sires for USDA carcass traits and palatability attributes of cooked ribeye steaks. *Journal of Animal Science* **78**, 1710–1715.

FRIEND, S.C. and STOCKDALE, P.H. (1980) Experimental Eimeria bovis infection in calves: a histopathological study. *Canadian journal of comparative medicine. Revue canadienne de médecine comparée* **44**, 129–40.

GUPTA, S. and EARLEY, B. (2005) Effect of repeated regrouping and relocation on the physiological, immunological, and hematological variables and performance of steers. *Journal of animal science*, 1948–1958.

JONSSON, N.N., PIPER, E.K., GRAY, C.P., DENIZ, A. and CONSTANTINOIU, C.C. (2011) Efficacy of toltrazuril 5 % suspension against Eimeria bovis and Eimeria zuernii in calves and observations on the associated immunopathology. *Parasitology research* **109**, S113–S128.

KANO, S., YOSHIDA, Y., TAKAHASHI, T., MATSUDA, K., HIDEYA, O. and ICHIJO, T. (2011) Relationship between proliferation of Clostridium perfringens in feces and recovery of fecal properties in Japanese Black calves with coccidiosis. *Journal of livestock medicine* **58**, 679–684.

LEBRUN, M., MAINIL, J.G. and LINDEN, A. (2010) Cattle enterotoxaemia and Clostridium perfringens : description , diagnosis and prophylaxis. doi:10.1136/vr.b4859.

LEVINE, N.D. (1985) Veterinary protozoology. Iowa State University Press.

LEVINE, N.D. and IVENS, V. (1967) The sporulated oocysts of Eimeria illinoisensis n. sp. and of other species of Eimeria of the ox. *Journal of Protozoology* **14**, 351–360.

MALOY, K.J. and POWRIE, F. (2011) Intestinal homeostasis and its breakdown in inflammatory bowel disease. *Nature* **474**, 298–306.

MATSUBAYASHI, M., KITA, T., NARUSHIMA, T., KIMATA, I., TANI, H., SASAI, K. and BABA, E. (2009) Coprological survey of parasitic infections in pigs and cattle in slaughterhouse in Osaka, Japan. *The Journal of veterinary medical science* **71**, 1079–83.

MCDOUGALD, L.R. (1998) Intestinal protozoa important to poultry. *Poultry Science* **77**, 1156–1158.

403 MENGEL, H., KRUGER, M., KRUGER, M.U., WESTPHAL, B., SWIDSINSKI, A.,
404 SCHWARZ, S., MUNDT, H.-C., DITTMAR, K. and DAUGSCHIES, A.
405 (2012) Necrotic enteritis due to simultaneous infection with Isospora suis and
406 clostridia in newborn piglets and its prevention by early treatment with
407 toltrazuril. *Parasitology research* **110**, 1347–55.

408 MUNDT, H.-C., BANGOURA, B., RINKE, M., ROSENBRUCH, M. and
409 DAUGSCHIES, A. (2005) Pathology and treatment of Eimeria zuernii
410 coccidiosis in calves: investigations in an infection model. *Parasitology*
411 *international* **54**, 223–30.

412 NADE, T., HIRABARA, S., OKUMURA, T. and FUJITA, K. (2003) Effects of
413 Vitamin A on Carcass Composition Concerning Younger Steer Fattening of
414 Wagyu Cattle. *Asian-Australasian Journal of Animal Sciences* **16**, 353–358.

415 ODA, K. and NISHIDA, Y. (1990) Prevalence and distribution of bovine coccidia in
416 Japan. *Japanese journal of veterinary science (Nihon Juigaku Zasshi)* **52**, 71–7.

417 OKA, A., MARUO, Y., MIKI, T., YAMASAKI, T. and SAITO, T. (1998) Influence
418 of vitamin A on the quality of beef from the Tajima strain of Japanese Black
419 cattle. *Meat science* **48**, 159–67.

420 POLKINGHORNE, R.J., NISHIMURA, T., NEATH, K.E. and WATSON, R. (2011)
421 Japanese consumer categorisation of beef into quality grades, based on Meat
422 Standards Australia methodology. *Animal Science Journal* **82**, 325–333.

423 SATO, A., ONOJIMA, M. and ONO, H. (2010) An outbreak of coccidiosis in the
424 period from the middle to the end of fattening stage in a Japanese cattle
425 fattening farm. *Journal of livestock medicine* **57**, 547–552.

426 STOCKDALE, P.H.G., BAINBOROUGH, A.R., BAILEY, C.B. and NIILO, L.
427 (1981) Some pathophysiological changes associated with infection of Eimeria
428 zuernii in calves. *Canadian journal of comparative medicine* **45**, 34–7.

429 SUHARA, S. and KANEI, M. (1992) Measurement of vitamin A. *Journal of medical*
430 *technology (Rinsho kensa)* **36**, 235–239.

431 SÜHWOLD, A., HERMOSILLA, C., SEEGER, T., ZAHNER, H. and TAUBERT, A.
432 (2010) T cell reactions of Eimeria bovis primary and challenge-infected calves.
433 *Parasitology research* **106**, 595–605.

434 SWANSON, J.C. and MORROW-TESCH, J. (2001) Cattle transport: Historical,
435 research, and future perspectives. *Journal of animal science*.
436 http://www.journalofanimalscience.org/content/79/E-Suppl/E102.full.pdf.
437 Accessed May 16, 2014.

438 TAYLOR, M.A. and CATCHPOLE, J. (1994) Coccidiosis of domestic ruminants.
439 *Applied parasitology* **35**, 73–86.

440 THIENPONT, D., ROCHETTE, F. and VANPARIJS, O.F.J. (1986) Diagnosing
441 Helminthiasis by Coprological Examination. 2nd ed. Janssen Research
442 Foundation, Beerse, Belgium.

443 VEISSIER, I. and BOISSY, A. (2001) Calves' responses to repeated social
444 regrouping and relocation. *Journal of animal science*, 2580–2593.

445 WESTPHAL, L.B., BERNEMANN, B. and KATHMANN, U.B. (2007) Isospora
446 suis- and Clostridium perfringens as mixed infection in suckling piglets just
447 after post partum? *Tierärztl Umschau* **62**, 682–689.

448 WU, S.-B., STANLEY, D., RODGERS, N., SWICK, R.A. and MOORE, R.J. (2014)
449 Two necrotic enteritis predisposing factors, dietary fishmeal and Eimeria

infection, induce large changes in the caecal microbiota of broiler chickens. *Veterinary microbiology* **169**, 188–97.

453 Table 1

454 Faecal score

455

Fecal consistency	Score
Normal to pasty with blood	1
Semi-liquid to liquid with blood	2
Watery with blood and/or tissue	3

456

457

458 **Figure legends**

459

460 Fig. 1

461 Age distribution of haemorrhagic enteritis cases in different age groups. The age

462 analysis was conducted only on animals in the fattening process (n=47).

463

464 Fig. 2

465 Comparison between Haemorrhagic Enteritis (HE) group and control group in the

466 faecal counts for *Eimeria* oocysts (A), coliforms (B) and *Clostridium perfringens*

467 (C). (A) Means of *Eimeria* oocyst count per gram of faeces in HE group (n=55)

468 and control group (n=21). *, $P<0.001$. (B) Enumeration of coliforms in the faeces

469 submitted for quantitative analysis. Results represent the means of CFU per gram

470 of faeces in the HE group (n=25) and control group (n=16). *, $P<0.001$. (C)

471 Bacterial count of *C. perfringens* in the faeces submitted for quantitative analysis.

472 Results represent the means of CFU per gram of faeces in the HE group (n=25)

473 and control group (n=16). $P=0.363$.

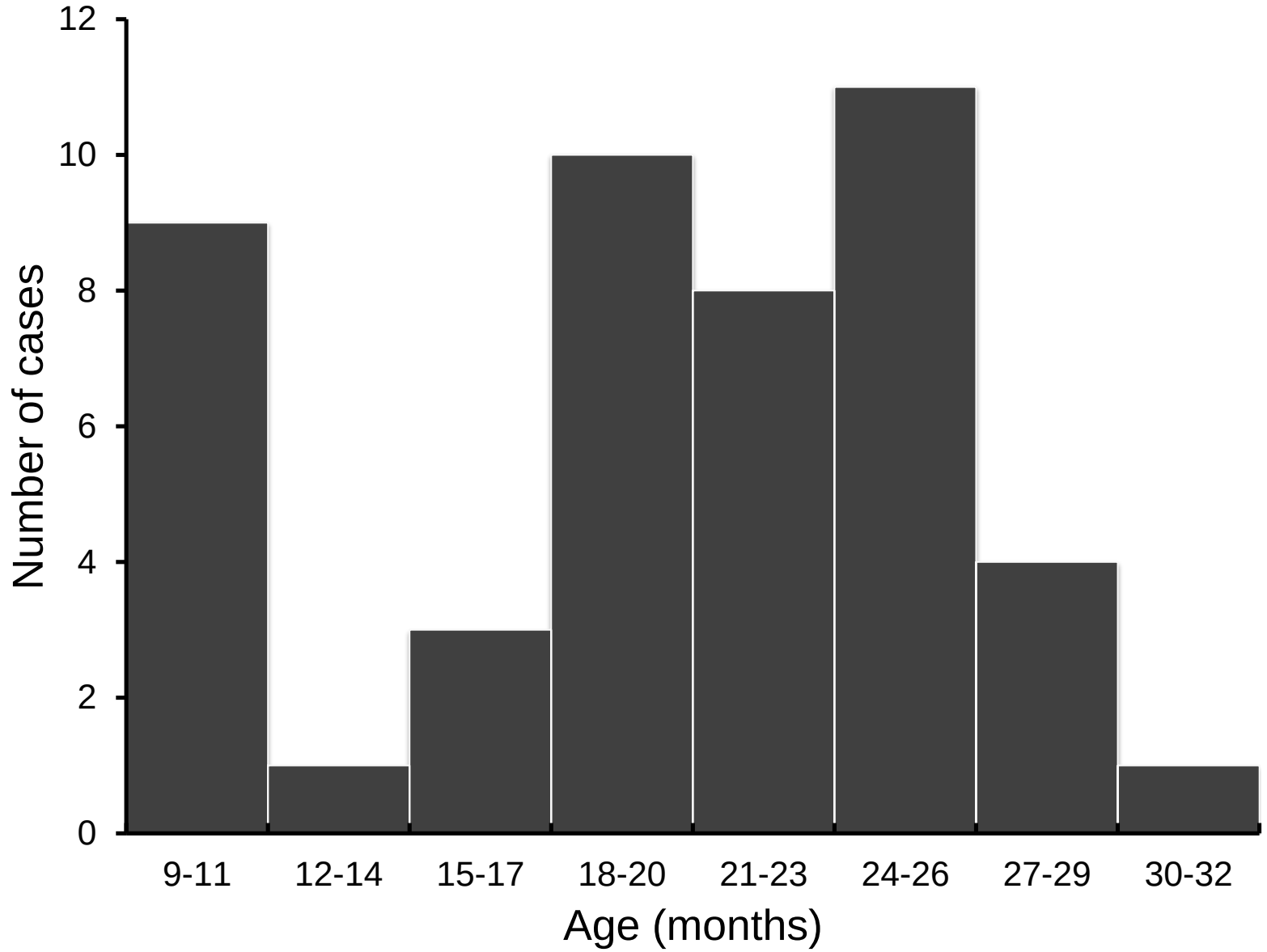
474

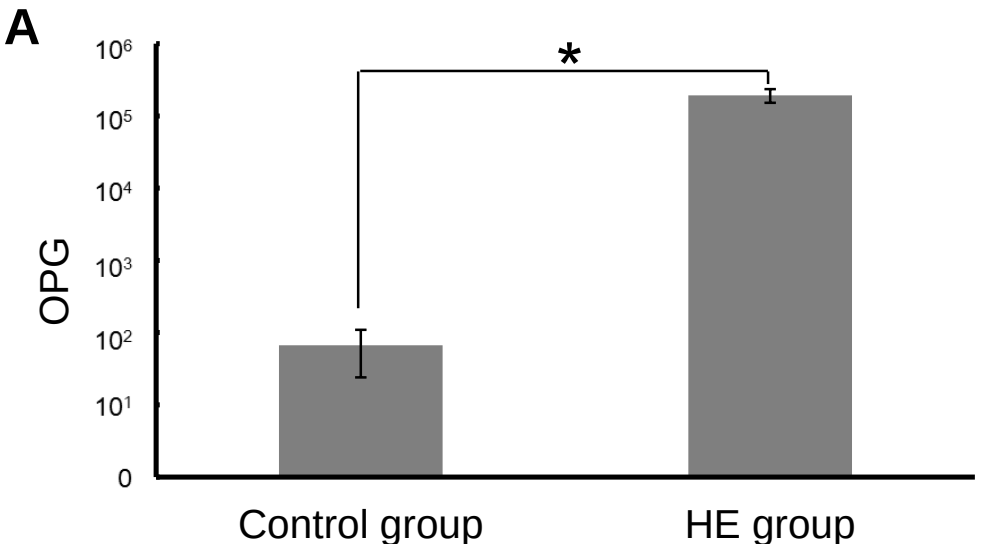
475 Fig. 3

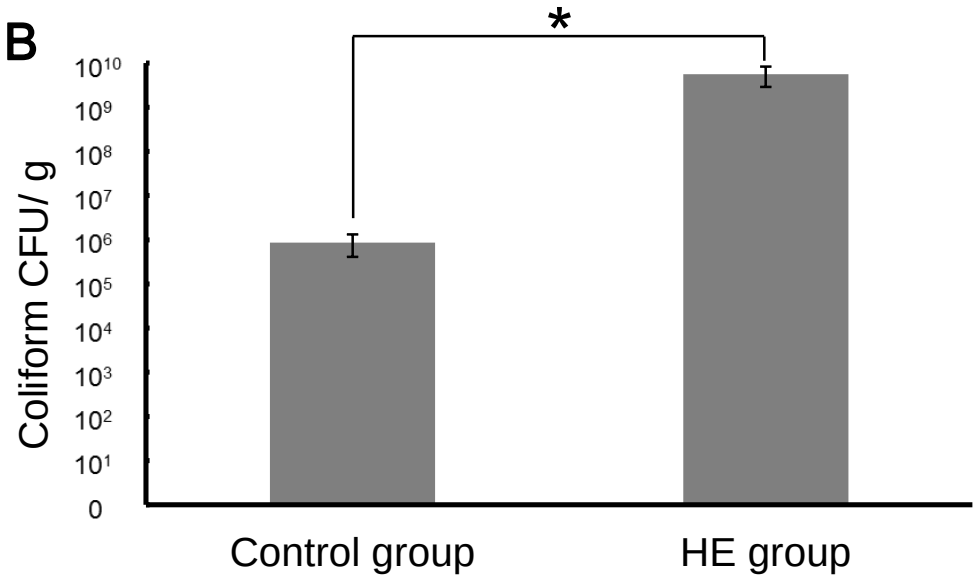
476 Proportion of each *Eimeria* species in OPG ranks. All of the animals were
477 classified according to their total OPG by ten-fold serial ranking and each bar
478 represents the result of each rank. The mean proportions of *Eimeria zuernii*, *E.*
479 *bovis* and other species (marked as "Others") are distinguished by different
480 patterns in each bar. The classification of "Others" consists of *E. auburnensis*, *E.*
481 *ellipsoidalis*, and *E. alabamensis* in decreasing order of frequency.
482
483 Fig. 4
484 Scatterplot graph showing the correlation between *Eimeria* OPG and *C.*
485 *perfringen*s bacterial count for each individual. Each data point represents one
486 individual. Filled circles represent individuals of the HE group and open circles
487 represent those of the control group. The trend line was generated from a total of
488 21 data points representing samples containing *C. perfringen*s.
489
490 Fig. 5
491 A case with a wine-like liquid stool, expressing celialgia and straining
492
493 Fig. 6
494 Mean total OPGs for each faecal score group. *, P=0.017.
495

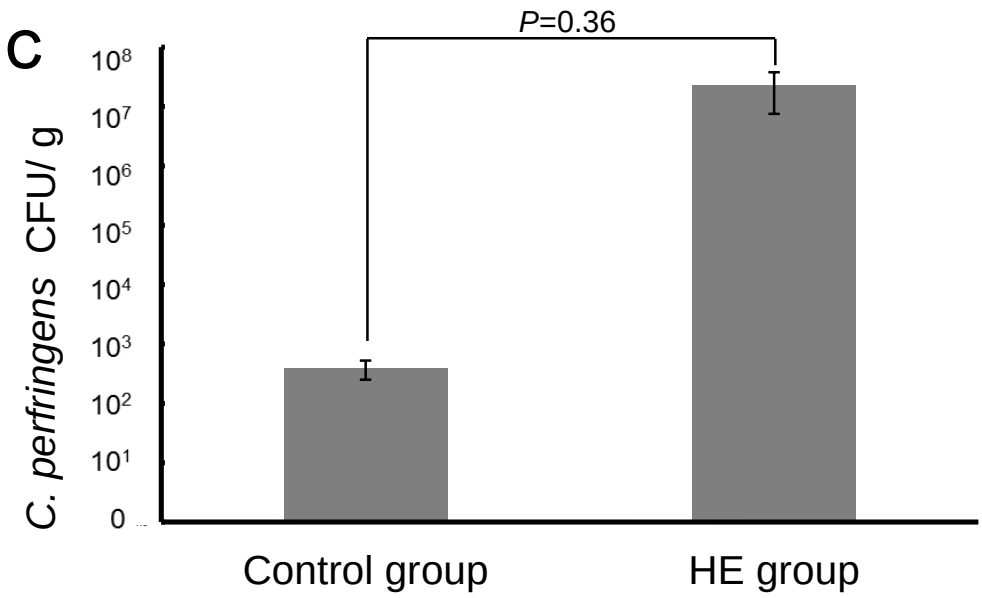
Fig. 7

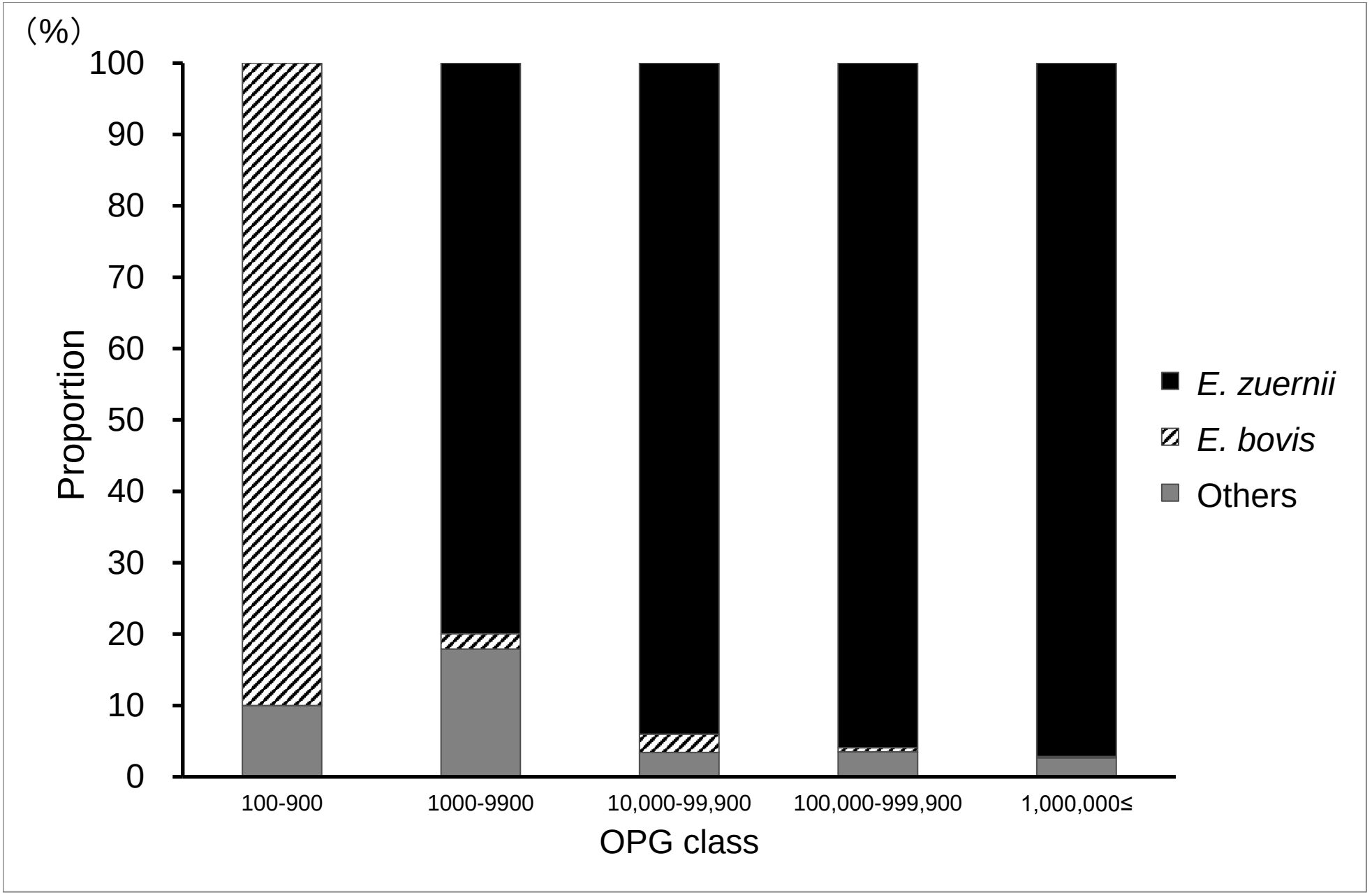
Scatterplot graph showing serum vitamin A concentrations of 40 head of fattening cattle in the HE group at different months of age. Lines show data from mass profile tests conducted for farm consultation in the study area. Broken line indicates the mean value of serum vitamin A concentration for each age and the solid line shows its standard deviations.

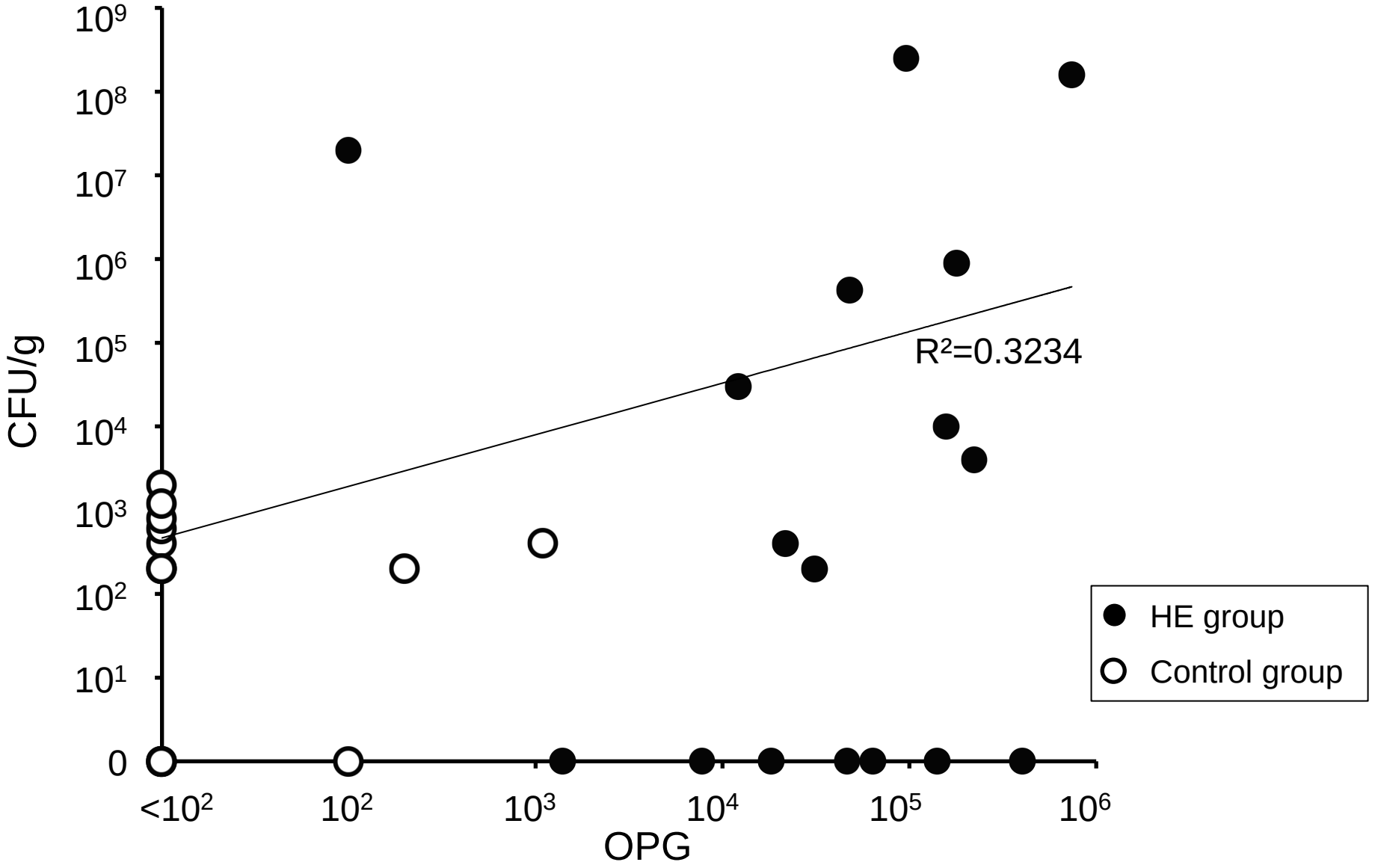

12
10
8
6
4
2
0
Number of cases
9-11
12-14
15-17
18-20
21-23
24-26
27-29
30-32
Age (months)

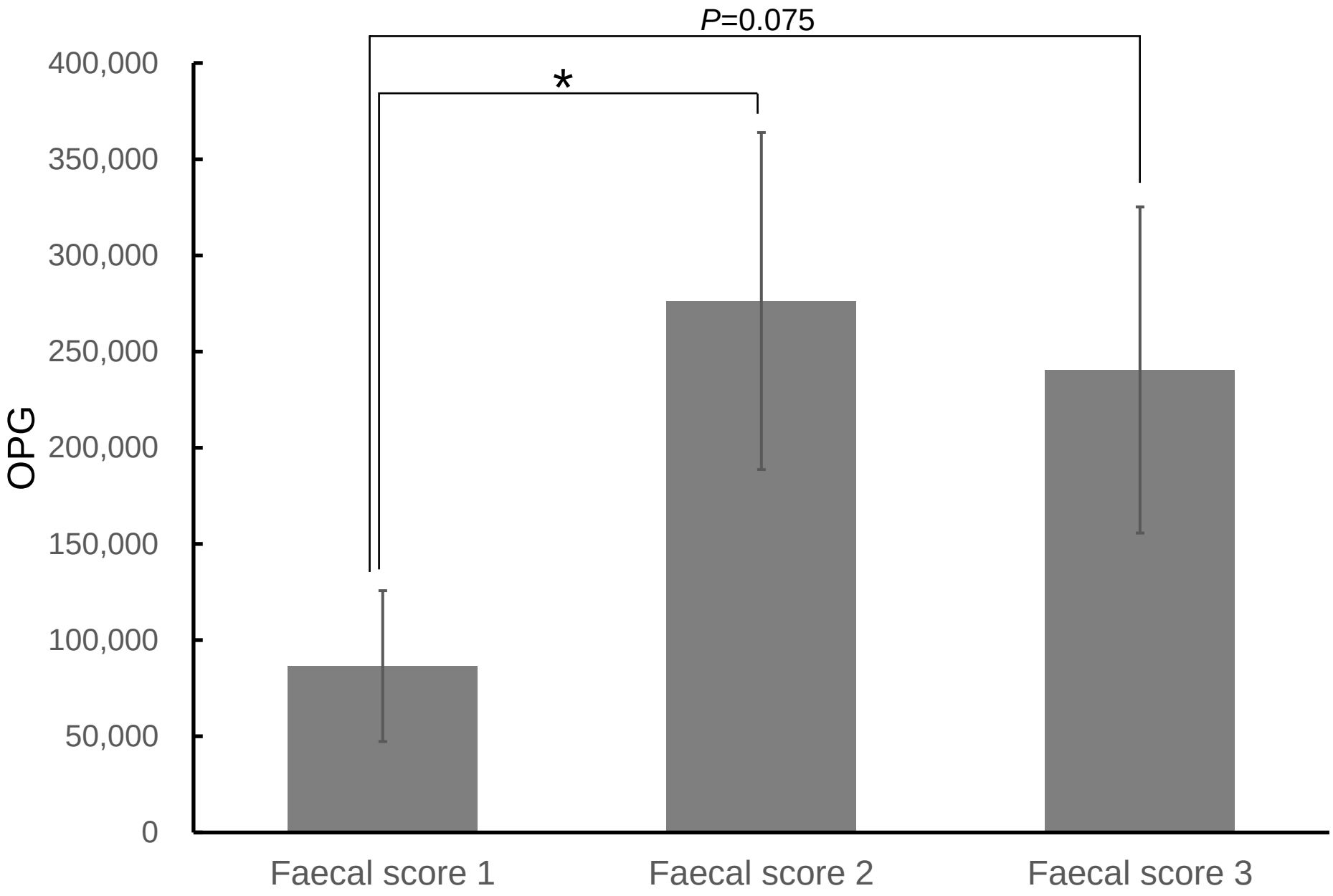

A
OPG
10^6
10^5
10^4
10^3
10^2
10^1
0
*
Control group
HE group

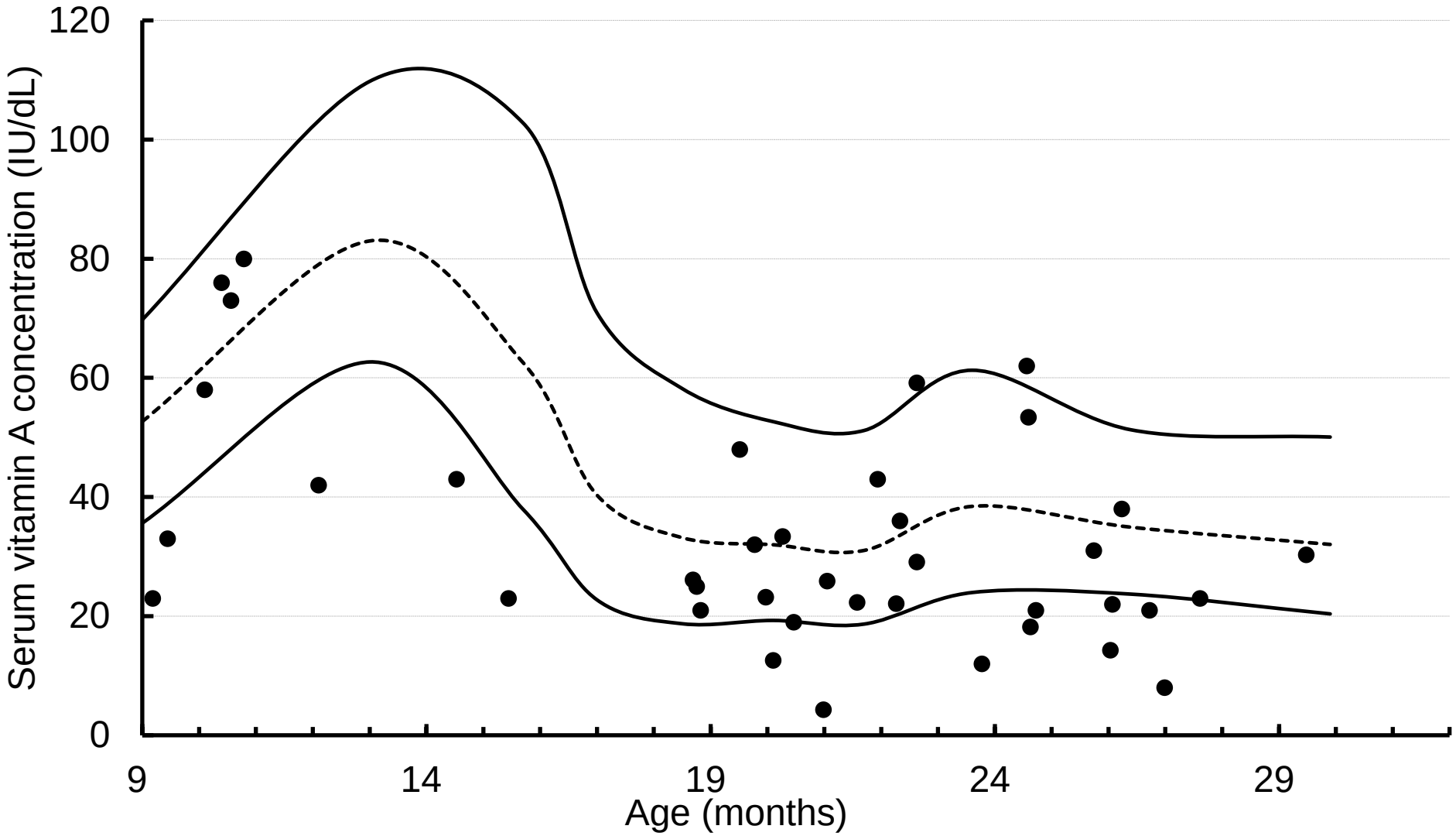

B
Coliform CFU/ g
10^{10}
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
0
*
Control group
HE group


C
C. perfringens CFU/ g
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
0
P=0.36
Control group
HE group


(%)
100
90
80
70
60
50
40
30
20
10
0
Proportion
100-900
1000-9900
10,000-99,900
100,000-999,900
1,000,000≤
OPG class
E. zuernii
E. bovis
Others


CFU/g
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
0
R^2=0.3234
HE group
Control group
<10^2
10^2
10^3
10^4
10^5
10^6
OPG


P=0.075
*
400,000
350,000
300,000
250,000
200,000
150,000
100,000
50,000
0
OPG
Faecal score 1
Faecal score 2
Faecal score 3


120
100
80
60
40
20
0
Serum vitamin A concentration (IU/dL)
9
14
19
24
29
Age (months)