Bifidobacterium longum BB536 supplementation is associated with increased circulating choline plasmalogen concentrations in non-pregnant, non-lactating dairy cows

Asato Yuuki[A], *Tomoaki Kubo*[B]*, *Maki Hashimoto*[C], *Takuji Wakatsuki*[B], *Haruna Sakamoto*[A], *Tamako Tanigawa*[B], *Shinichi Kitamura*[C], *Hiroya Kadokawa*[A]

[A] *Faculty of Veterinary Medicine, Yamaguchi University, Yamaguchi-shi, Yamaguchi-ken 1677-1, Japan*
Tel.: +81 83 9335825; Fax: +81 83 9335938

[B] *Dairy Cattle Group, Dairy Research Center, Hokkaido Research Organization, Nakashibetsu, Hokkaido, 086-1135, Japan*

[C] *Organization for Research Promotion, Osaka Metropolitan University, 1-2 Gakuen-cho, Naka-ku, Sakai, Osaka 599-8531, Japan.*

[*] *Asato Yuuki and Tomoaki Kubo contributed equally to this work.*

[*] *Correspondence to: Hiroya Kadokawa*
Faculty of Veterinary Medicine, Yamaguchi University, Yamaguchi-shi, Yamaguchi-ken 1677-1, Japan
Tel.: +81 83 9335825; Fax: +81 83 9335938
E-mail address: hiroya@yamaguchi-u.ac.jp

Running head: B. longum and circulating plasmalogens

ABSTRACT

Context: Plasmalogens are ether phospholipids implicated in neuroendocrine regulation, including reproductive function. Recent studies suggest that circulating plasmalogen concentrations are associated with reproductive performance in dairy cows; however, practical strategies to increase these levels remain limited.

Aims: We hypothesised that supplementation with *Bifidobacterium longum* increases circulating choline plasmalogen concentrations and that this response depends on physiological state.

Methods: Commercial probiotic products were screened using liquid chromatography–mass spectrometry and metagenomics to identify candidates containing plasmalogen-producing bacteria. A product containing the characterised strain *B. longum* BB536 and products containing other *B. longum* strains were selected for *in vivo* evaluation. Selected products were administered to Holstein cattle, and circulating choline plasmalogen concentrations were measured using an enzyme-based fluorometric assay.

Key results: In long-term non-pregnant, non-lactating dairy cows, supplementation with *B. longum* BB536 significantly increased circulating choline plasmalogen concentrations, with a detectable rise approximately one week after the start of treatment and peak levels during days 8–14 ($P < 0.05$). In contrast, no consistent increase was observed in pregnant, lactating dairy cows. Cross-sectional analysis across pregnancy stages revealed significant variation in circulating choline plasmalogen concentrations ($P < 0.05$), with lower concentrations during mid- to late gestation. No adverse effects were observed in ruminal pH, blood lactate concentrations, or body weight.

Conclusion: These findings suggest that supplementation with *B. longum* BB536 increases circulating choline plasmalogen concentrations in a state-dependent manner.

Implications: This study provides new insight into the regulation of plasmalogens in cattle and suggests a potential nutritional approach for modulating reproductive function.

Keywords: Bifidobacterium longum BB536, choline plasmalogens, dairy cows, GPR61, pregnancy, probiotics, reproduction, ruminants

Introduction

Plasmalogens are a class of ether phospholipids that are particularly abundant in the brain and are involved in a wide range of physiological functions (Almsherqi 2021; Li et al. 2022; Liu et al. 2020). These lipids have been implicated in the prevention of neurodegenerative disorders such as Alzheimer's disease (Almsherqi 2021; Li et al. 2022; Liu et al. 2020). In addition, maternal transfer of plasmalogens via milk has been suggested to support the development of the neonatal nervous system (Ramadurai et al. 2022). Structurally, plasmalogens are characterised by a vinyl ether bond at the sn-1 position, an ester-linked fatty acid at the sn-2 position, and either choline or ethanolamine as the head group at the sn-3 position (Braverman and Moser 2012). In cattle, choline plasmalogens are the predominant form in circulation, whereas ethanolamine plasmalogens are enriched in the brain (Kadokawa et al. 2021; Saito et al. 2023).

Our group has reported a potential mechanism by which plasmalogens regulate reproductive function. Both ethanolamine plasmalogens and choline plasmalogens bind to the orphan receptor GPR61 expressed in anterior pituitary gonadotrophs and stimulate the secretion of FSH and LH independently of gonadotropin-releasing hormone (Pandey et al. 2017; Kereilwe et al. 2018; Kadokawa et al. 2022; Kadokawa et al. 2025). Furthermore, we have shown that circulating plasmalogen concentrations decrease around parturition in dairy cows, and that early recovery of these levels is associated with improved reproductive performance and milk production (Saito et al. 2023). These findings suggest that circulating plasmalogen concentrations are linked to reproductive function in dairy cattle.

In humans and experimental animals, dietary plasmalogens can be absorbed through intestinal lymphatic pathways and distributed via the circulation (Nishimukai et

al. 2003). However, direct supplementation with plasmalogens is costly and may not be practical for routine use in livestock production systems. Accordingly, alternative strategies to increase endogenous or microbially derived plasmalogens are of interest.

Some studies have suggested that *Bifidobacterium longum* may produce plasmalogens (Mawatari et al. 2018; Mawatari et al. 2020). Among these, *B. longum* BB536 is a well-characterised probiotic strain originally developed for human use, registered in the American Type Culture Collection (ATCC BAA-999), and granted Generally Recognized As Safe (GRAS) status by the U.S. Food and Drug Administration. This strain has been widely used in probiotic products for human consumption and studied in multiple regions, including North America, Europe, and Asia. However, direct evidence that administration of such bacteria increases circulating plasmalogen concentrations *in vivo* remains limited, particularly in large animals. Therefore, we hypothesised that supplementation with *B. longum* BB536 increases circulating choline plasmalogen concentrations in dairy cows. We further examined whether this response differs according to physiological status, particularly pregnancy and lactation.

Materials and Methods

Selection of probiotic products

Prior to the *in vivo* feeding experiment in cattle, commercially available probiotic products in Japan were screened to identify candidates containing plasmalogen-producing bacteria.

Plasmalogen fractions were first extracted from each product according to a previously reported method (Saito et al. 2023). The presence of plasmalogens was then analysed using two-dimensional liquid chromatography–mass spectrometry, as described

previously (Takahashi et al. 2018). Plasmalogens were identified based on characteristic retention behaviour and mass spectral features, including diagnostic fragment ions.

Subsequently, metagenomics was conducted to confirm the presence of plasmalogen-producing bacteria. The V1–V9 regions of the 16S rRNA gene were analysed using long-read amplicon sequencing (PacBio Revio platform; Bioengineering Lab Co., Ltd., Kanagawa, Japan). Taxonomic assignment was performed based on sequence similarity to reference databases.

Experiment in long-term non-pregnant, non-lactating Holstein dairy cows

All experiments were performed in accordance with the Guiding Principles for the Care and Use of Experimental Animals in the Field of Physiological Sciences (Physiological Society of Japan) and were approved by the Committee on Animal Experiments of Yamaguchi University (Approval No. 652) and the Hokkaido Research Organization (Approval No. 20250519-09).

The animal experiment was conducted at the Dairy Research Center, Hokkaido Research Organization (Nakashibetsu, Hokkaido, Japan). A total of 12 Holstein dairy cows that had been non-pregnant and non-lactating for at least 36 months (parity: 1.3 ± 0.5; age: 7.9 ± 0.3 years) were used. These animals were intentionally maintained in a non-lactating, non-pregnant state, rather than being in a conventional dry period associated with late pregnancy, to exclude potential effects of pregnancy. Animals were allocated to treatment groups to balance age and parity across groups.

All animals were fed twice daily with a basal total mixed ration (TMR) for the dry period (Table 1) during a 1-week pre-treatment period, followed by a 2-week treatment period and a 1-week post-treatment period according to the Japanese feeding standard

(Agriculture, Forestry and Fisheries Research Council Secretariat, 2017). During the treatment period, animals received one of the following probiotic products: P (paste form; 10 mL once daily by oral administration; Bio-Pro Paste for Cattle; Protocol Japan Co., Ltd., Hokkaido, Japan), D (powder form; 30 g placed on top of the feed twice daily; Bio-Pro DFM; Protocol Japan Co., Ltd.), or B (capsule form; four capsules placed on top of the feed twice daily; Morinaga Bifidus; Morinaga Milk Industry Co., Ltd., Tokyo, Japan). Products P and D were manufactured by Protocol Naturals (Bridgeport, TX, USA). The dosage of each product was determined according to the manufacturer's instructions and adjusted to provide comparable colony-forming units across treatments.

Feed was provided twice daily at 10:00 and 16:00. Daily feed intake ranged from 18 to 22 kg per animal, adjusted according to body weight, and no feed refusals were observed during the experiment. Blood samples were collected four times per week via the jugular vein into heparinised tubes. Plasma was separated by centrifugation and stored at −30 °C until analysis.

Rumen fluid was collected twice weekly using a rumen fluid sampler (model 16132000, Fujihira Industry Co., Ltd., Tokyo, Japan) to monitor ruminal pH. Blood lactate concentrations were measured using a portable analyser (Lactate Pro 2; Arkray Inc., Tokyo, Japan). Body weight was recorded once weekly.

Experiment in pregnant, lactating dairy cows

To examine whether the effect of *B. longum* BB536 depends on physiological status, a separate experiment was conducted in pregnant, lactating dairy cows. A total of five Holstein dairy cows were used and fed a TMR ad libitum during the lactation period (Table 1). Animals were maintained under the same housing and feeding conditions as

described for the non-pregnant, non-lactating cow experiment. All animals were in their second or third parity (parity: 2.4 ± 0.2; age: 3.6 ± 0.4 years) and were at 76.5 ± 21.4 days of gestation and 216.4 ± 15.5 days after the previous parturition. All animals were fed the TMR during a 2-week pre-treatment period, followed by a 2-week treatment period and a 2-week post-treatment period. This longer pre- and post-treatment design was adopted to account for greater physiological variability in lactating cows. The probiotic formulation, dosage, and administration protocol were identical to those used in the long-term non-pregnant, non-lactating cow experiment described above. Briefly, probiotic product B (*B. longum* BB536) was administered as eight capsules per day (four capsules twice daily) by top-dressing onto the TMR twice daily during the treatment period. Blood sampling, plasma preparation, and measurement of circulating choline plasmalogen concentrations were performed as described above.

Cross-sectional analysis of pregnancy stage

To examine the effect of pregnancy stage on circulating plasmalogen concentrations, a cross-sectional analysis was conducted using independent animals at different physiological stages. Holstein dairy cows in their second or third parity (parity: 2.3 ± 0.1; age: 3.6 ± 0.7 years) at various pregnancy stages (1–9 months), as well as non-pregnant (Pre-AI) and recently calved (Fresh) cows, were included in the analysis. The number of animals in each group ranged from 5 to 9, depending on the stage. Blood samples were collected via the jugular vein into heparinised tubes, and plasma was separated by centrifugation and stored at −30 °C until analysis. Circulating choline plasmalogen concentrations were measured using the same enzyme-based fluorometric assay as described above.

Measurement of circulating choline plasmalogen concentrations

Circulating choline plasmalogen concentrations were measured using an enzyme-based fluorometric assay developed by our group (Saito et al. 2023). All organic solvents used were of HPLC grade (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). In the present study, only choline plasmalogens were measured because the enzyme reagent required for ethanolamine plasmalogen quantification was not available.

Plasmalogen fractions were extracted from plasma samples using acidified buffer and organic solvent extraction, followed by nitrogen drying and reconstitution in assay buffer, as described previously (Mawatari et al. 2018). The assay is based on the enzymatic hydrolysis of choline plasmalogens by phospholipase D, releasing choline. Choline is then oxidized by choline oxidase to generate hydrogen peroxide, which reacts with Amplex Red in the presence of peroxidase to produce the fluorescent compound resorufin. Fluorescence intensity was measured using a microplate reader (ARVO X4, PerkinElmer, Waltham, MA, USA) (excitation at 540 nm, emission at 615 nm), and concentrations were calculated from standard curves. The specificity of the assay is high, as other choline-containing phospholipids do not produce a detectable fluorescent signal (Morita et al. 2020). The limit of detection was 2.18 μg/mL. At 2 mg/mL, the intra- and inter-assay coefficient of variation were 0.3 % and 0.5 %, respectively.

Statistical analysis

Data were analysed using StatView Ver. 5.0 for Windows (SAS Institute Inc., NC, USA).

For the experiment in long-term non-pregnant, non-lactating Holstein dairy cows,

the effects of treatment and time on circulating choline plasmalogen concentrations, ruminal pH, blood lactate concentration, and body weight were evaluated using repeated-measures analysis of variance (ANOVA). When significant effects were detected, post hoc comparisons were performed using the Bonferroni test. The interaction between treatment and time was also evaluated. In addition, changes from baseline (day 0) within each treatment group were evaluated using paired *t*-tests.

For the experiment in pregnant, lactating dairy cows, changes from baseline (day 0) were evaluated using paired *t*-tests.

For the experiment examining pregnancy stage-dependent changes in circulating plasmalogen concentrations, homogeneity of variance was assessed using the Brown–Forsythe test. As the assumption of equal variances was violated ($P < 0.05$), differences among groups were analysed using Welch's one-way ANOVA, followed by the Games–Howell test for multiple comparisons.

Statistical significance was defined as $P < 0.05$, and data are presented as mean ± standard error of the mean (SEM).

Results

Selection of probiotic products

Among the commercially available probiotic products screened, only three products (designated P, D, and B) were identified as containing detectable plasmalogens and were therefore selected for further analysis. Products P and D were derived from the same manufacturer and differed only in formulation (paste and powder, respectively).

Metagenomic analysis based on long-read sequencing of the V1–V9 regions of the 16S rRNA gene revealed marked differences in microbial composition among the

228 selected products. Product B was labelled as containing *B. longum* BB536, which was
229 consistent with the metagenomic results showing the presence of *B. longum* as the sole
230 bacterial species.

231 In contrast, products P and D contained mixed bacterial populations. The
232 dominant species was *Lactobacillus acidophilus* (67.6%), followed by *B. longum* (17.4%)
233 and *Enterococcus faecium* (14.8%). Minor bacterial species (<0.2%) included *Bacillus*
234 *subtilis, Lactobacillus plantarum, Vibrio metschnikovii, Kocuria polaris, Microcystis*
235 *wesenbergii, Adhaeribacter aquaticus, Truepera radiovictrix,* and Bog-
236 515_sp003155135.

237

238 *Effects of probiotic administration on circulating choline plasmalogen concentrations in*
239 *long-term non-pregnant, non-lactating dairy cows*

240 Among the three probiotic products tested (P, D, and B), only product B significantly
241 increased circulating choline plasmalogen concentrations (Fig. 1). Repeated-measures
242 ANOVA revealed a significant effect of treatment ($P < 0.01$) and a significant treatment
243 × time interaction ($P < 0.05$). Post hoc Bonferroni tests showed that concentrations in the
244 B group were significantly higher than those in the P and D groups during the treatment
245 period ($P < 0.05$). In the B group, circulating choline plasmalogen concentrations
246 increased approximately one week after the start of treatment and remained elevated
247 during the treatment period. During days 8–14, mean values in the B group were
248 approximately 1.5-fold higher than those in the P and D groups ($P < 0.05$). Following the
249 cessation of treatment, concentrations in the B group declined gradually rather than
250 abruptly. In contrast, no marked changes were observed in the P or D groups throughout
251 the experimental period.

252

253 *Effects of probiotic administration on rumen pH, blood lactate concentration, and body*
254 *weight in long-term non-pregnant, non-lactating dairy cows*

255 Rumen pH remained stable throughout the experimental period, with values
256 generally ranging between 6.5 and 7.2 across all treatment groups (Fig. 2A). Blood lactate
257 concentrations remained within the normal range (approximately 0.5–3.0 mM) and
258 showed no marked changes following probiotic administration (Fig. 2B). No signs of
259 lactic acidosis were observed. Body weight remained stable in all groups, with values
260 ranging from approximately 870 to 950 kg, and no significant changes were detected
261 during the experimental period (Fig. 2C).

262 These results indicate that probiotic supplementation was not associated with
263 detectable adverse effects under the conditions of the present study.

264

265 *Effects of B. longum BB536 administration on circulating choline plasmalogen*
266 *concentrations in pregnant, lactating dairy cows*

267 In contrast to the results observed in non-pregnant, non-lactating cows, treatment
268 with *B. longum* BB536 was not associated with a significant increase in circulating
269 choline plasmalogen concentrations in pregnant, lactating dairy cows (Fig. 3). Circulating
270 choline plasmalogen concentrations gradually decreased over the experimental period,
271 and no significant increase was detected during or after the treatment period.

272

273 *Effects of B. longum BB536 administration on rumen pH, blood lactate concentration,*
274 *and body weight in pregnant, lactating dairy cows*

275 Rumen pH, blood lactate concentrations, and body weight remained stable

276 throughout the experimental period, with no significant changes observed (Fig. 4A–C).
277 These results are consistent with those observed in long-term non-pregnant, non-lactating
278 dairy cows.
279
280 *Changes in circulating choline plasmalogen concentrations across pregnancy stages*
281 To examine whether physiological status influences circulating plasmalogen
282 concentrations, a cross-sectional analysis was conducted using independent animals at
283 different stages. Circulating choline plasmalogen concentrations differed significantly
284 among stages (Welch's ANOVA, $P < 0.01$; Fig. 5). Concentrations exhibited stage-
285 dependent variation, with lower values during mid- to late gestation. Notably,
286 concentrations at months 8 and 9 of gestation were significantly lower than those at month
287 1 (Games–Howell test, $P < 0.05$). As these data were obtained from independent animals
288 at each stage, they reflect differences between stages rather than temporal changes within
289 individuals.
290
291 **Discussion**
292 The present study showed that supplementation with a probiotic containing *B.*
293 *longum* BB536 was associated with increased circulating choline plasmalogen
294 concentrations in long-term non-pregnant, non-lactating dairy cows, whereas no such
295 increase was observed in pregnant dairy cows. In addition, circulating choline
296 plasmalogen concentrations varied across pregnancy stages. These findings suggest that
297 circulating plasmalogen levels are not solely determined by dietary or microbial input,
298 but are regulated according to physiological status.
299 Among the probiotic products examined, only product B significantly increased

300 circulating choline plasmalogen concentrations. This product contained *B. longum*
301 BB536 as a single bacterial strain, whereas the other products contained mixed bacterial
302 populations. However, because the *B. longum* strains present in products P and D were
303 not fully characterised, the observed effect cannot be attributed solely to *B. longum*
304 BB536. Previous studies have shown that plasmalogens are present in *B. longum* but not
305 in closely related species such as *B. animalis* (Mawatari et al. 2020). However, whether
306 ingestion of such bacteria increases circulating plasmalogen concentrations *in vivo* has
307 not been established. The present findings therefore suggest that specific gut bacteria can
308 contribute to the regulation of circulating plasmalogen levels in cattle.

309 Plasmalogens are biologically important lipids in mammals. In humans, they are
310 associated with protection against neurodegenerative diseases (Almsherqi 2021; Li et al.
311 2022; Liu et al. 2020). In dairy cows, circulating plasmalogen concentrations decrease
312 markedly after parturition, and early recovery is associated with improved reproductive
313 performance and milk production (Saito et al. 2023). Circulating plasmalogen
314 concentrations are also positively correlated with FSH and anti-Müllerian hormone
315 concentrations, suggesting a role in ovarian function (Saito et al. 2023). In addition,
316 plasmalogens stimulate gonadotropin secretion from bovine anterior pituitary cells
317 (Pandey et al. 2017; Kereilwe et al. 2018; Kadokawa et al. 2022; Kadokawa et al. 2025).
318 These findings indicate that increasing circulating plasmalogen concentrations may
319 contribute to improved reproductive performance in dairy cattle. Further studies are
320 required to determine whether *B. longum* BB536 supplementation is effective in
321 postpartum cows.

322 In contrast to the results observed in long-term non-pregnant, non-lactating dairy
323 cows, no significant increase in circulating plasmalogen concentrations was detected

following *B. longum BB536* supplementation in pregnant dairy cows. Because the experimental design and dosage were identical, this difference is unlikely to be due to methodological factors. Instead, it suggests that physiological status may markedly influence plasmalogen dynamics. The cross-sectional analysis further supports this interpretation, showing that circulating plasmalogen concentrations were lower during mid- to late pregnancy. In our previous study, plasma choline plasmalogen concentrations were longitudinally measured in individual cows and were shown to decrease around parturition, followed by an increase and subsequent stabilisation near the first postpartum ovulation (Saito et al. 2023). Notably, the present cross-sectional data showed a similar pattern across physiological stages, with lower concentrations in Fresh cows and higher, stabilised concentrations in Pre-AI cows. Although the current dataset does not represent longitudinal changes within the same individuals, the consistency between these cross-sectional observations and previous longitudinal data suggests that the observed stage-dependent variation reflects an underlying physiological trajectory. However, because this analysis was based on independent animals at each stage, these data should be interpreted as an estimate of stage-dependent variation rather than a direct longitudinal change within individuals.

Plasmalogens are absorbed via intestinal lymphatic pathways in mammals (Nishimukai et al. 2003), and a similar mechanism is likely to operate in cattle. One possible explanation for the lack of response in pregnant cows is that circulating plasmalogens may be increasingly utilised for foetal development, particularly in the developing brain, where high levels are required (Braverman and Moser 2012; Farooqui and Horrocks 2001). Alterations in brain plasmalogen composition during prenatal development have been shown to affect neural function and behaviour in rodents (Hino

et al. 2019). In addition, plasmalogen-containing phospholipids have been detected in maternal, placental and foetal compartments, and their levels appear to change under different maternal conditions (Powell et al. 2023). Alternatively, the increased metabolic demands associated with pregnancy and lactation may enhance the utilisation or redistribution of plasmalogens to peripheral tissues. However, direct evidence for plasmalogen transfer to the foetus is currently limited, and the underlying mechanisms remain unclear.

The present findings also provide insight into the behaviour of *B. longum* BB536 in the bovine digestive tract. Bifidobacteria are generally considered to exhibit host specificity, with distinct species associated with different hosts. Comparative genomic analyses have shown that bifidobacteria have adapted to host-specific glycan environments (Milani et al. 2015; Milani et al. 2017). In cattle, the digestive system differs markedly from that of humans, consisting of a multi-compartment stomach including the rumen, which harbours a diverse microbial ecosystem. These features are expected to limit the survival and colonisation of exogenous bifidobacteria.

Nevertheless, the present results suggest that *B. longum* BB536 may transiently survive and exert functional effects in the bovine intestine. Bifidobacteria are obligate anaerobes that require simple carbohydrates and amino acids, and therefore are unlikely to substantially proliferate in the rumen. This interpretation is consistent with the absence of changes in ruminal pH and blood lactate concentrations. Instead, *B. longum* BB536 may persist in the distal small intestine or proximal colon, where nutrient conditions are generally more favorable (Duranti et al. 2020; Rivière et al. 2016; Turroni et al. 2018). Because plasmalogens are absorbed via intestinal lymphatic pathways, bacterial activity in these regions could contribute to the observed increase in circulating plasmalogen

372 concentrations.

373 Only product B increased circulating plasmalogen concentrations, suggesting that
374 the observed effect depends on specific properties of the probiotic strain. However,
375 differences in formulation (e.g. capsule versus paste or powder) and interactions with
376 other bacterial species cannot be excluded as contributing factors. Further studies are
377 required to distinguish among these possibilities.

378 Our previous study (Saito et al. 2023) demonstrated that circulating plasmalogen
379 concentrations decline with ageing in dairy cows. Although direct evidence in cattle
380 remains limited, age-related alterations in gut microbial composition have been widely
381 reported in other species, including humans and rodents (O'Toole and Jeffery 2015;
382 Claesson et al. 2012). Therefore, similar age-related microbial changes may also occur in
383 cattle and contribute to the observed decline in circulating plasmalogen levels. In this
384 context, the present findings, showing that *B. longum* BB536 supplementation increases
385 circulating plasmalogen concentrations in a physiological state–dependent manner,
386 provide a basis for further investigation into a potential gut microbiota–plasmalogen axis
387 in bovine physiology.

388 The present study has several limitations. First, because the probiotic products
389 differed not only in bacterial composition but also in formulation (e.g. capsule versus
390 paste or powder), the observed effects cannot be attributed solely to *B. longum* BB536.
391 Second, the strains of *B. longum* present in products P and D were not fully characterised,
392 and therefore strain-specific effects cannot be excluded. Third, the cross-sectional
393 analysis of pregnancy stages was based on independent animals rather than longitudinal
394 measurements within individuals, which limits the interpretation of temporal changes.
395 Fourth, the underlying mechanisms linking gut microbial activity and circulating

plasmalogen concentrations were not examined directly. Fifth, reproductive hormones such as LH, FSH, and estradiol were not measured. Because the non-pregnant, non-lactating cows were not synchronized for estrous cycles and blood samples were collected once daily, interpretation of single-point hormonal measurements would have been limited, particularly for pulsatile hormones such as LH. Finally, the pregnant, lactating cow experiment was conducted using a small sample size (n = 5). A formal a priori power analysis was not performed because this study was exploratory in nature and constrained by animal availability. Therefore, modest treatment effects may not have been detected, and the negative findings in pregnant, lactating dairy cows should be interpreted with caution. Further studies are required to clarify these processes, including microbial dynamics, intestinal absorption, systemic distribution of plasmalogens, endocrine responses, and reproductive outcomes.

It is noteworthy that *B. longum* BB536, a strain originally isolated from the human infant intestine and developed for human use, may exert detectable effects in the bovine gastrointestinal environment. Although this may appear unexpected given host specificity, the strain has been selected as a human probiotic for its ability to survive gastrointestinal transit and reach the intestine in a viable form. *B. longum* BB536 is also one of the most extensively studied Bifidobacterium strains, with a wide range of reported health benefits in humans (Hidalgo-Cantabrana et al. 2017). In addition, clinical studies have demonstrated beneficial effects of *B. longum* BB536 in humans, including improved bowel function (Takeda et al. 2023) and modulation of immune-related outcomes in maternal–infant settings (Enomoto et al. 2014). Therefore, the present findings raise the possibility that *B. longum* BB536 may have broader applications in cattle under specific physiological conditions, although further studies are required to evaluate these effects.

Conclusion

In conclusion, supplementation with a probiotic containing *B. longum* BB536 was associated with increased circulating choline plasmalogen concentrations in long-term non-pregnant, non-lactating dairy cows, whereas no significant increase was detected in pregnant dairy cows. Together with previous longitudinal findings, these results suggest that circulating plasmalogen concentrations follow a regulated physiological trajectory associated with reproductive status. The present study extends these observations to pregnancy, demonstrating stage-dependent variation during gestation. These findings suggest that circulating plasmalogen levels are dynamically regulated according to physiological status and highlight the potential of probiotic strategies to modulate plasmalogen levels in livestock, with possible implications for nutritional strategies to support reproductive performance in dairy cattle.

Data availability

The data that support this study will be shared upon reasonable request to the corresponding authors.

Conflicts of interest

The authors declare no conflicts of interest.

Declaration of funding

This research was partly supported by a Grant-in-Aid for Scientific Research [JSPS Kakenhi grant number 24K01910 and 25K22421] from the Japan Society for the Promotion of Science (Tokyo, Japan) to Hiroya Kadokawa. The funder had no role in the study design, data collection and analysis, decision to publish, and preparation of the manuscript.

Acknowledgements

The authors thank Caetano Kaempf Farret, a trainee of the Japan International Cooperation Agency (JICA), for assistance with blood sampling. The authors also thank the staff of the Dairy Research Center, Hokkaido Research Organization, for animal care and management.

Author contributions

H.K. conceived and supervised the study. Y.A., T.K., T.W., H.S. and T.T. performed the animal experiments. M.H. and S.K. conducted plasmalogen analyses. H.K. drafted the manuscript. All authors reviewed and approved the final manuscript.

References

Agriculture, Forestry and Fisheries Research Council Secretariat (2017) Nutrition requirement. In: 'Japanese feeding standard for dairy cattle'. (Eds National Agriculture and Food Research Organization) pp. 4-107. (Japan Livestock Industry Association, Tokyo, Japan) (in Japanese)

Almsherqi ZA (2021) Potential role of plasmalogens in the modulation of biomembrane morphology. *Frontiers in Cell and Developmental Biology* **9**, 673917. doi:10.3389/fcell.2021.673917

Braverman NE, Moser AB (2012) Functions of plasmalogen lipids in health and disease. *Biochimica et Biophysica Acta (BBA)* **1822**, 1442–1452. doi:10.1016/j.bbadis.2012.05.008

Claesson MJ, Jeffery IB, Conde S, Power SE, O'Connor EM, Cusack S, Harris HM, Coakley M, Lakshminarayanan B, O'Sullivan O, Fitzgerald GF, Deane J, O'Connor M,

Harnedy N, O'Connor K, O'Mahony D, van Sinderen D, Wallace M, Brennan L, Stanton C, Marchesi JR, Fitzgerald AP, Shanahan F, Hill C, Ross RP, O'Toole PW (2012) Gut microbiota composition correlates with diet and health in the elderly. *Nature* **488,** 178–184. doi:10.1038/nature11319

Duranti S, Longhi G, Ventura M, van Sinderen D, Turroni F (2020) Exploring the ecology of Bifidobacteria and their genetic adaptation to the mammalian gut. *Microorganisms* **9**, 8. doi: 10.3390/microorganisms9010008.

Enomoto T, Sowa M, Nishimori K, Shimazu S, Yoshida A, Yamada K, Furukawa F, Nakagawa T, Yanagisawa N, Iwabuchi N, Odamaki T, Abe F, Nakayama J, Xiao JZ (2014) Effects of bifidobacterial supplementation to pregnant women and infants in the prevention of allergy development in infants and on fecal microbiota. *Allergology international* **63**, 575-585. doi: 10.2332/allergolint.13-OA-0683.

Farooqui AA, Horrocks LA (2001) Plasmalogens: workhorse lipids of membranes in normal and injured neurons and glia. *Neuroscientist* 7, 232-245. doi: 10.1177/107385840100700308.

Hidalgo-Cantabrana C, Delgado S, Ruiz L, Ruas-Madiedo P, Sánchez B, Margolles A (2017) Bifidobacteria and their health-promoting effects. *Microbiology Spectrum* **5**, 10.1128/microbiolspec.bad-0010-2016. doi:10.1128/microbiolspec.BAD-0010-2016

Hino K, Kaneko S, Harasawa T, Kimura T, Takei S, Shinohara M, Yamazaki F, Morita SY, Sato S, Kubo Y, Kono T, Setou M, Yoshioka M, Fujino J, Sugihara H, Kojima H, Yamada N, Udagawa J (2019) Change in brain plasmalogen composition by exposure to prenatal undernutrition leads to behavioral impairment of rats. *Journal of Neuroscience* **39**, 7689-7702. doi: 10.1523/JNEUROSCI.2721-18.2019.

Kadokawa H, Kotaniguchi M, Kereilwe O, Kitamura S (2021) Reduced gonadotroph stimulation by ethanolamine plasmalogens in old bovine brains. *Scientific Reports* **11**, 4757. doi: 10.1038/s41598-021-84306-6.

Kadokawa H, Yoshino R, Saito R, Hirokawa T (2022) Chemosynthetic ethanolamine plasmalogen stimulates gonadotropin secretion from bovine gonadotrophs by acting as a potential GPR61 agonist. *Animal Reproduction Science* **241,** 106992. doi: 10.1016/j.anireprosci.2022.106992.

Kadokawa H, Niyonzima YB, Hirokawa T, Yoshino R (2025) Effects of chemosynthetic choline plasmalogens on gonadotropin secretion from bovine gonadotrophs. *Journal of Reproduction and Development* **71**, 201-209. doi: 10.1262/jrd.2025-019.

Kereilwe O, Pandey K, Kadokawa H (2018) Influence of brain plasmalogen changes on gonadotropin secretion from the cultured bovine anterior pituitary cells. *Domestic Animal Endocrinology* **64**, 77-83. doi:10.1016/j.domaniend.2018.04.002

Li K, Bertrand K, Naviaux JC, Monk JM, Wells A, Wang L, Lingampelly SS, Naviaux RK, Chambers C (2023) Metabolomic and exposomic biomarkers of risk of future neurodevelopmental delay in human milk. *Pediatric Research* **93**, 1710-1720. doi:10.1038/s41390-022-02283-6

Liu Z, Li C, Pryce J, Rochfort S (2020) Comprehensive characterization of bovine milk lipids: phospholipids, sphingolipids, glycolipids, and ceramides. *Journal of Agricultural and Food Chemistry* **68**, 6726-6738. doi:10.1021/acs.jafc.0c01604

Mawatari S, Hazeyama S, Morisaki T, Fujino T (2018) Enzymatic measurement of ether phospholipids in human plasma after hydrolysis of plasma with phospholipase A1. *Practical Laboratory Medicine* **10**, 44-51. doi:10.1016/j.plabm.2018.01.003

518 Mawatari S, Ohara S, Taniwaki Y, Tsuboi Y, Maruyama T, Fujino T (2020) Improvement
519 of blood plasmalogens and clinical symptoms in Parkinson's disease by oral
520 administration of ether phospholipids: a preliminary report. *Parkinson's Disease* **2020**,
521 2671070. doi:10.1155/2020/2671070

522 Milani C, Turroni F, Duranti S, Lugli GA, Mancabelli L, Ferrario C, van Sinderen D,
523 Ventura M (2015) Genomics of the genus Bifidobacterium reveals species-specific
524 adaptation to the glycan-rich gut environment. *Applied and Environmental*
525 *Microbiology* **82**, 980–991. doi:10.1128/AEM.03500-15

526 Milani C, Duranti S, Bottacini F, Casey E, Turroni F, Mahony J, Belzer C, Delgado
527 Palacio S, Arboleya S, Mancabelli L, Lugli GA, Rodriguez JM, Bode L, de Vos W,
528 Gueimonde M, Margolles A, van Sinderen D, Ventura M (2017) The first microbial
529 colonizers of the human gut: composition, activities, and health implications of the
530 infant gut microbiota. *Microbiology and Molecular Biology Reviews* **81**, e00036-17.
531 doi:10.1128/MMBR.00036-17

532 Morita SY, Tsuji T, Terada T (2020) Protocols for enzymatic fluorometric assays to
533 quantify phospholipid classes. *International Journal of Molecular Sciences* **21**, 1032.
534 doi:10.3390/ijms21031032

535 Nishimukai M, Wakisaka T, Hara H (2003) Ingestion of plasmalogen markedly increased
536 plasmalogen levels of blood plasma in rats. *Lipids* **38**, 1227-1235. doi:10.1007/s11745-
537 003-1183-9

538 O'Toole PW, Jeffery IB (2015) Gut microbiota and aging. *Science* **350**, 1214–1215.
539 doi:10.1126/science.aac8469

Pandey K, Kereilwe O, Borromeo V, Kadokawa H (2017) Heifers express G-protein coupled receptor 61 in anterior pituitary gonadotrophs in stage-dependent manner. *Animal Reproduction Science* **181**, 93-102. doi:10.1016/j.anireprosci.2017.03.020

Powell TL, Uhlson C, Madi L, Berry KZ, Chassen SS, Jansson T, Ferchaud-Roucher V (2023) Fetal sex differences in placental LCPUFA ether and plasmalogen phosphatidylethanolamine and phosphatidylcholine contents in pregnancies complicated by obesity. *Biology of Sex Differences* **14**, 66. doi: 10.1186/s13293-023-00548-1.

Ramadurai S, Andrews C, Cheema S, Thomas R, Wagner CL, Sen S (2022) Maternal predictors of breast milk plasmalogens and associations with infant body composition and neurodevelopment. *Clinical therapeutics* **44**, 998-1009. doi: 10.1016/j.clinthera.2022.06.003.

Rivière A, Selak M, Lantin D, Leroy F, De Vuyst L (2016) Bifidobacteria and butyrate-producing colon bacteria: importance and strategies for their stimulation in the human gut. *Frontiers in Microbiology* **7**, 979. doi:10.3389/fmicb.2016.00979

Saito R, Kubo T, Wakatsuki T, Asato Y, Tanigawa T, Kotaniguchi M, Hashimoto M, Kitamura S, Kadokawa H (2023~~a~~) Dynamic changes and importance of plasma concentrations of ether phospholipids, of which the majority are plasmalogens, in postpartum Holstein dairy cows. *Reproduction, Fertility and Development* **35**, 622-639. doi: 10.1071/RD23057.

Takeda T, Asaoka D, Nojiri S, Yanagisawa N, Nishizaki Y, Osada T, Koido S, Nagahara A, Katsumata N, Odamaki T, Xiao JZ, Ohkusa T, Sato N (2023) Usefulness of Bifidobacterium longum BB536 in elderly individuals with chronic constipation: a

563 randomized controlled trial. *The American Journal of Gastroenterology* **118**, 561–568.
564 doi:10.14309/ajg.0000000000002028

565 Takahashi R, Nakaya M, Kotaniguchi M, Shojo A, Kitamura S (2018) Analysis of
566 phosphatidylethanolamine, phosphatidylcholine, and plasmalogen molecular species in
567 food lipids using an improved 2D high-performance liquid chromatography system.
568 *Journal of Chromatography. B, Analytical Technologies in the Biomedical and Life*
569 *Sciences* **1077-1078**, 35-43. doi:10.1016/j.jchromb.2018.01.014

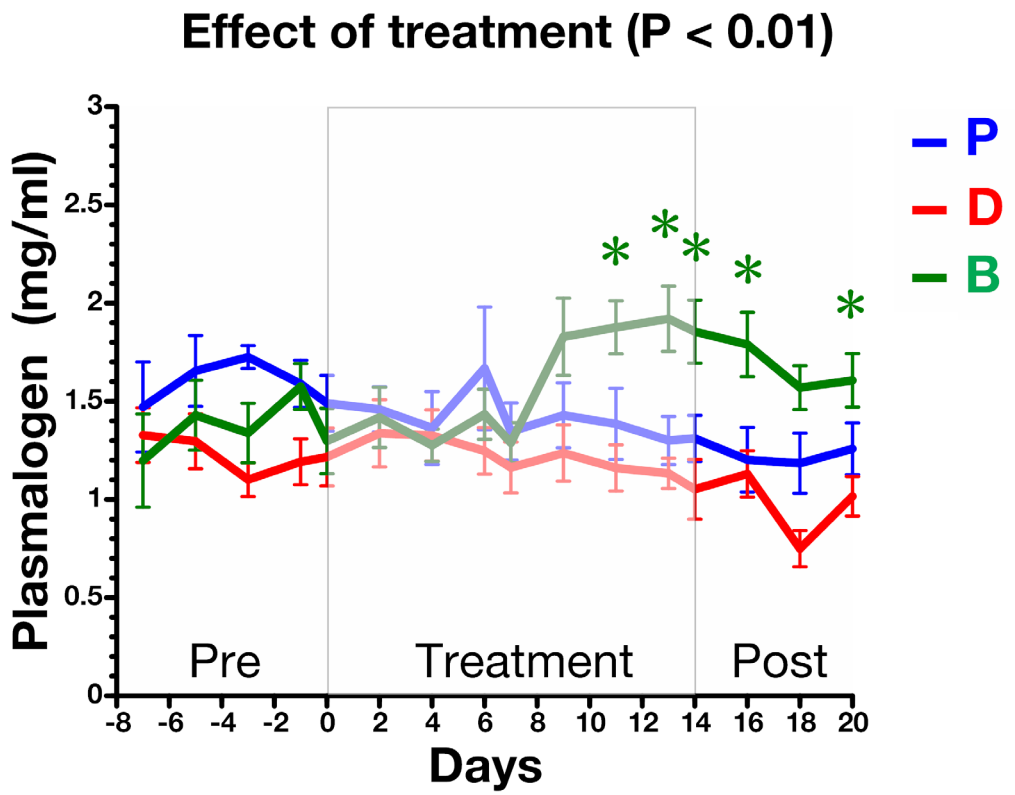
570 Turroni F, Milani C, Duranti S, Ferrario C, Lugli GA, Mancabelli L, van Sinderen D,
571 Ventura M (2018) Bifidobacteria and the infant gut: an example of co-evolution and
572 natural selection. *Cellular and Molecular Life Sciences* **75**, 103-118. doi:
573 10.1007/s00018-017-2672-0.
574

575 **Table 1.** Formulation and chemical composition of the total mixed ration (TMR).
576 Ingredient proportions are expressed on a dry matter basis and presented as means ± SD
577 of the TMR prepared during each experimental period.

	Dry period (%)	**Lactation period** (%)
Ingredients (% dry matter)		
Grass silage	33.0 ± 0.3	31.0 ± 0.2
Corn silage	24.2 ± 0.1	20.0 ± 0.1
Corn grain, steam-flaked	24.2 ± 0.2	25.0 ± 0.1
Soybean meal	12.1 ± 0.1	17.0 ± 0.1
Beet pulp, dry, molasses added	5.5 ± 0.1	5.5 ± 0.1
Calcium carbonate	1.1 ± 0.01	1.6 ± 0.01
Chemical composition		
Dry matter (%)	41.7 ± 0.3	34.4 ± 0.2
Metabolizable energy (Mcal/kg)	2.61 ± 0.01	2.77 ± 0.01
Crude protein (% dry matter)	15.1 ± 0.04	16.6 ± 0.02
Neutral detergent fibre (% dry matter)	37.9 ± 0.1	37.8 ± 0.1
Non-fibre carbohydrate (% dry matter)	36.1 ± 0.1	35.0 ± 0.1
Organic matter (% dry matter)	93.6 ± 0.02	93.2 ± 0.01
Ether extract (% dry matter)	4.5 ± 0.03	4.2 ± 0.02

578
579

580 **Figure Legends**



581

582 **Fig. 1.** Changes in circulating plasmalogen concentrations following administration of

583 probiotic products (P, D, and B) in long-term non-pregnant, non-lactating Holstein dairy

584 cows. Product B (*B. longum* BB536) was associated with increased plasmalogen

585 concentrations approximately one week after the start of treatment, and elevated levels

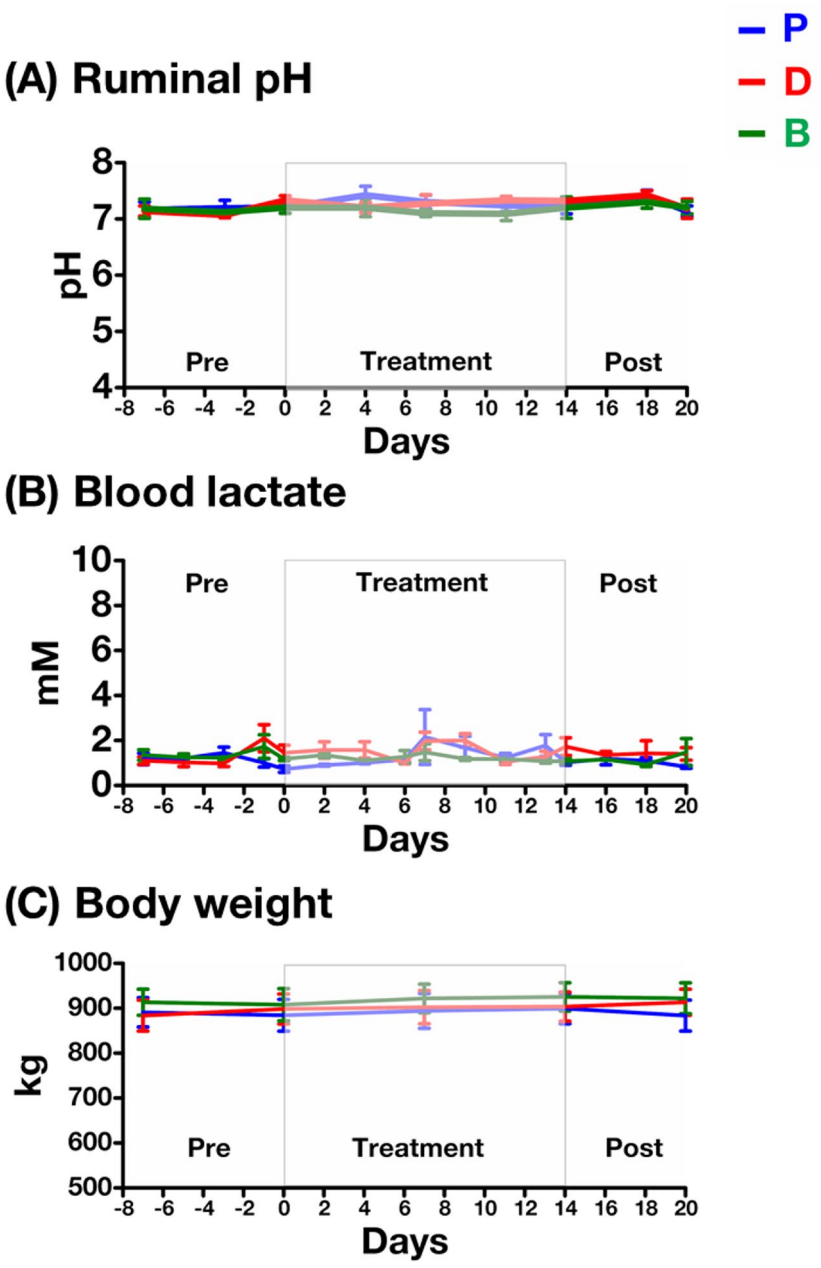
586 were maintained during the treatment period. No marked changes were observed in

587 animals treated with products P or D. Repeated-measures ANOVA revealed a significant

588 effect of treatment ($P < 0.01$). Post hoc Bonferroni tests indicated significant differences

589 between product B and the other groups ($P < 0.05$). Values are presented as mean ± SEM

590 ($n = 4$ per group). *$P < 0.05$ vs. day 0.



591

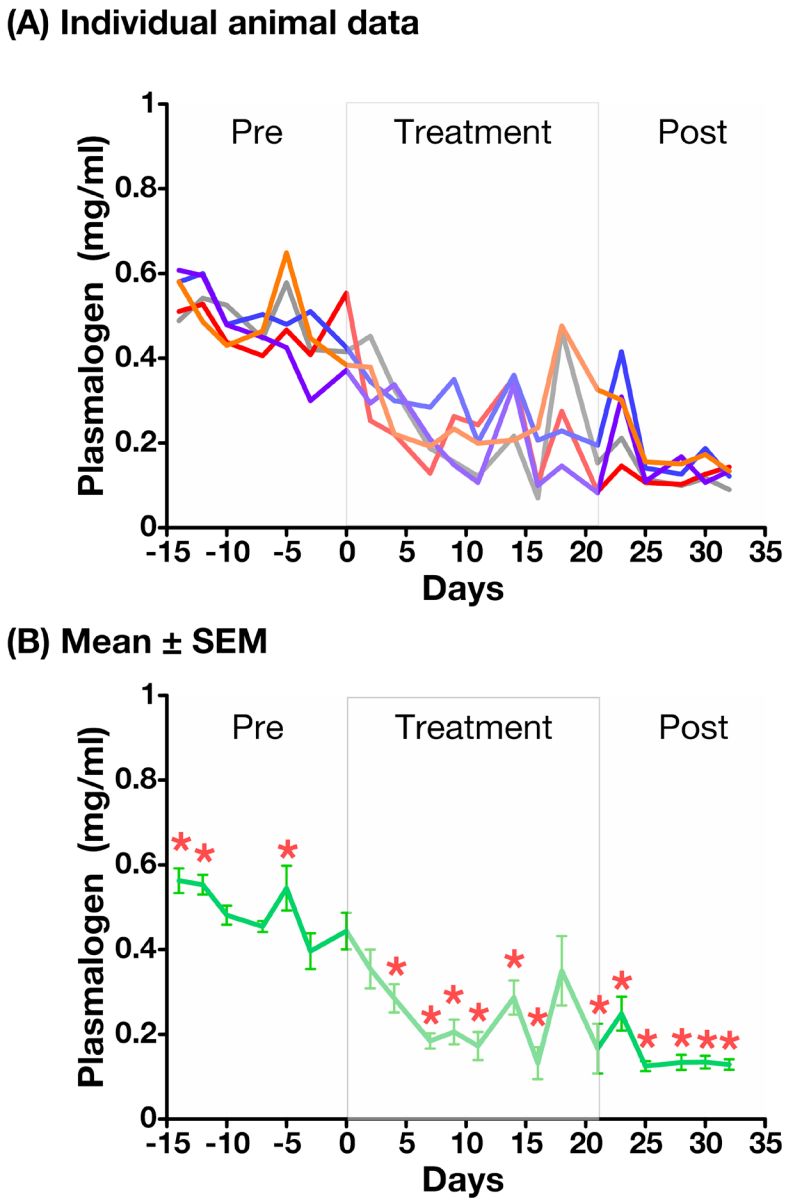
592 **Fig. 2.** Changes in ruminal pH (A), blood lactate concentrations (B), and body weight (C)

593 following probiotic administration in long-term non-pregnant, non-lactating Holstein

594 dairy cows. All parameters remained within normal ranges and showed no significant

595 changes during the experimental period. Values are presented as mean ± SEM (n = 4 per

596 group).



597
598

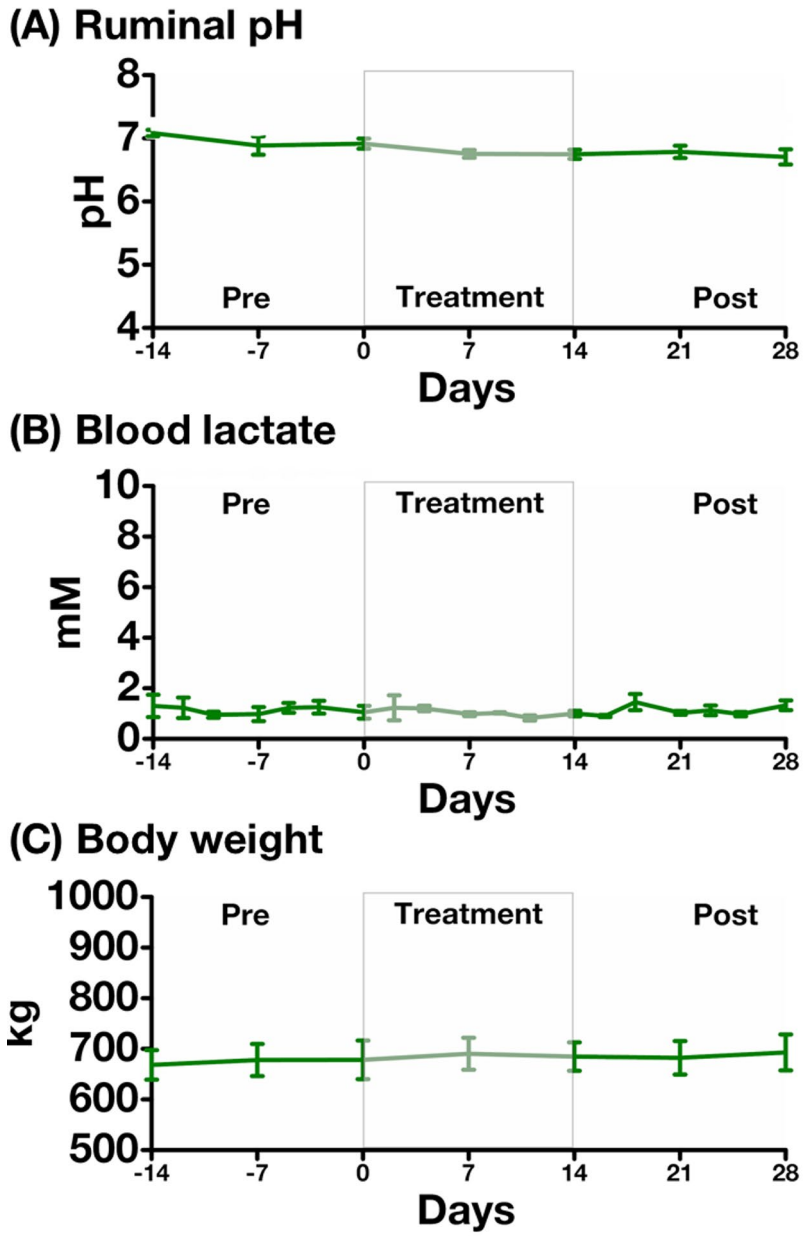
599 **Fig. 3.** Changes in circulating choline plasmalogen concentrations following

600 supplementation with *B. longum* BB536 in pregnant, lactating dairy cows. (A) Individual

601 animal data (n = 5). (B) Mean ± SEM (n = 5). The shaded area indicates the treatment

602 period. Red asterisks indicate significant differences compared with day 0 ($P < 0.05$).

603



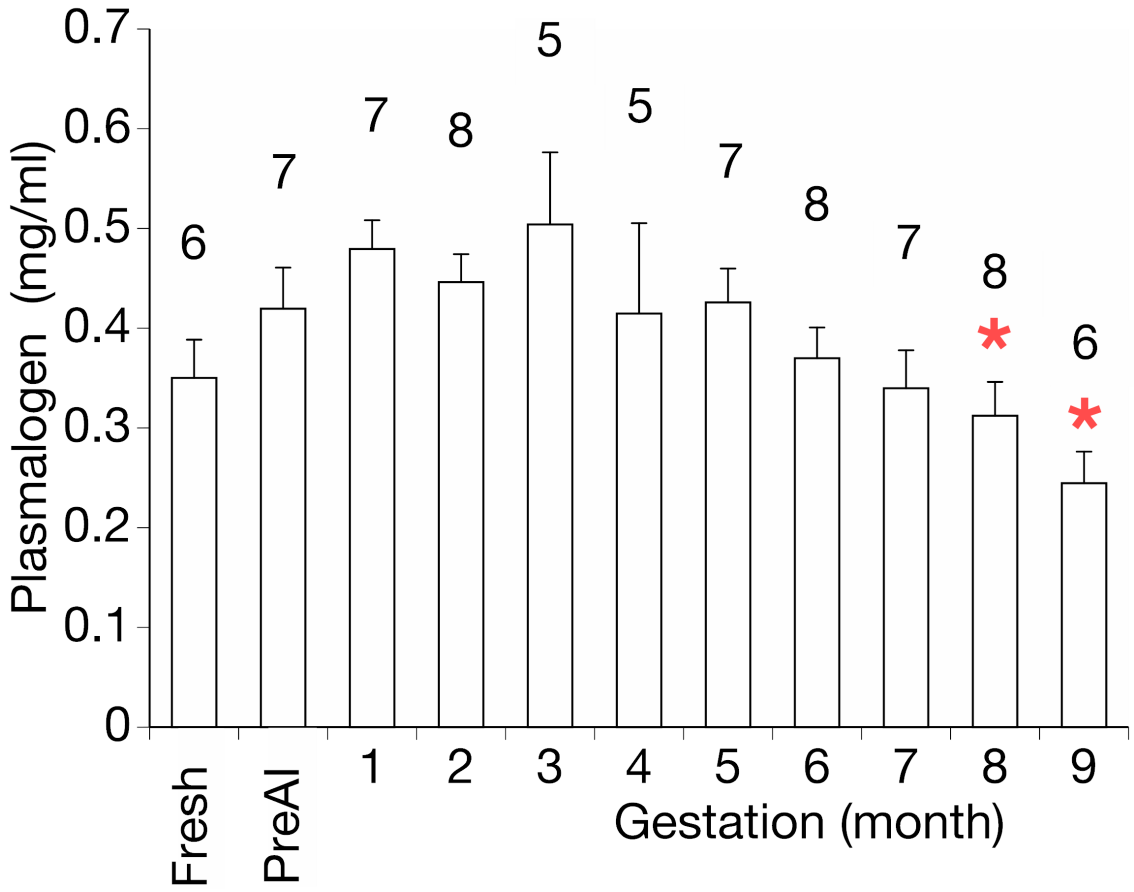
604

605 **Fig. 4.** Changes in ruminal pH (A), blood lactate concentrations (B), and body weight (C)

606 following supplementation with *B. longum* BB536 in pregnant, lactating dairy cows. No

607 significant changes were detected over time in any parameter. Values are presented as

608 mean ± SEM (n = 5).



609

610 **Fig. 5.** Stage-dependent variation in circulating choline plasmalogen concentrations in

611 dairy cows. Fresh indicates within 1 month after parturition, and Pre-AI indicates more

612 than 1 month after parturition and before the first artificial insemination. Numbers 1–9

613 represent months of gestation. Columns represent the mean ± SEM, with sample sizes

614 indicated above the columns. Red asterisks indicate significant differences compared with

615 month 1 of gestation ($P < 0.05$, Games–Howell test).