Effect of supplementing sodium butyrate, phytogenic compounds and egg yolk antibodies in calf milk replacer containing probiotic bacteria on selected faecal bacteria in calves

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ABSTRACT. Thirty two Holstein calves were used to investigate the effects of sodium butyrate (SB), phytogenic compounds (PC; consisting mainly of caraway, liquorice extract, oak bark and vanilla flavour), and egg yolk antibodies (EY) supplementation in milk replacer (MR) containing probiotic bacteria on the counts of total bacteria and selected bacteria in faeces. The calves were fed a mixture of surplus colostrum, transition milk and whole milk for the first 9 days of life. On day 10, they were assigned to one of four treatments (8 calves/treatment): 1) MR containing probiotic bacteria but no other feed additives (control treatment; CTRL); 2) MR with SB; 3) MR with PC; and 4) MR with EY. On day 14 of the study, total bacteria counts were higher in EY calves, *Lactobacillus* spp. counts were higher in PC and EY calves, and *Bifidobacterium* spp. counts were higher in SB, PC and EY calves compared to CTRL calves. *Escherichia coli* counts were higher in EY calves and tended to be higher in SB calves compared to CTRL. On day 28 of the study, total bacteria counts tended to be lower in PC calves, *Lactobacillus* spp. counts were higher, while *Clostridium perfringens* counts were lower in EY calves compared to CTRL calves. It was concluded that the supplementation of SB, PC and EY in MR already containing probiotic bacteria may either increase or reduce the abundance of beneficial (*Lactobacillus* spp. and *Bifidobacterium* spp.) but also undesired (*E. coli*, and *C. perfringens*) bacteria in calf faeces. Of the investigated feed additives, PC supplementation exerted the most beneficial effect on the composition of faecal bacteria, while EY supplementation can reduce *C. perfringens* shedding in faeces.

Introduction

Probiotics, prebiotics, phytogenic compounds and butyrate sources are commonly used feed additives in calf milk replacers (MR) (Frieten et al., 2017; Stefańska et al., 2021; Jahani-Azizabadi et al., 2022). Among these feed additives, probiotic bacteria are most often included in calf MR formulas due to their well-established positive effect on calf growth and health, as confirmed by the results of numerous studies (Signorini et al., 2012; Cangiano et al., 2020). One of the mechanisms through

which probiotics exert their beneficial impacts involves modulating the intestinal microbiome. Probiotic bacteria can colonise the gastrointestinal tract (GIT) and enhance settlement of other beneficial bacteria (Plaza-Diaz et al., 2019), thereby reducing the probability of GIT colonisation by pathogenic bacteria. Numerous studies have demonstrated that feeding probiotics to calves increases the counts of beneficial bacteria (e.g. *Lactobacillus* spp.) and reduces the counts of harmful or potentially harmful bacteria (e.g. *Escherichia coli*) in faeces (Roodposhti and Dabiri, 2012; Signorini et al., 2012). Consequently, calves receiving probiotic feed additives often experience fewer episodes of diarrhoea (Signorini et al., 2012; Cangiano et al., 2020).

The gastrointestinal microbiome in newborn ruminants is not only affected by feeding probiotic bacteria, but also by other feed additives. For example, sodium butyrate supplementation in MR was shown to affect microbiota composition in the colon of calves (O'Hara et al., 2018), nucleotide addition to MR affected *Lactobacillus* spp. counts in the faeces of calves (Górka et al., 2021), while egg yolk antibodies, derived from eggs laid by immunised hens, reduced the shedding of certain pathogens in the faeces of lambs (Cook et al., 2005). Moreover, pathogenic compound supplementation in calf MR was demonstrated to affect rumen bacterial population (Jahani-Azizabadi et al., 2022) and reduce the occurrence and shedding of *Cryptosporidium* and *Giardia duodenalis* in faeces (Stefańska et al., 2021). Of the aforementioned feed additives, knowledge regarding the effects of phytogenic additives in calf MR is particularly limited. This is due to the wide variation in composition of different phytogenic additives, which depend on the specific plant materials or extracts and their combinations in the formulation. It has also been repeatedly observed that the doses of this type of feed additives are critical for their final effects, as excessive doses may lead to negative outcomes (Brand et al., 2019; Kolif et al., 2021; Jahani-Azizabadi et al., 2022). Therefore, further research is needed to fill the gap in our understanding of the effects of phytogenic feed additives supplementation in calf MR.

Probiotic bacteria are commonly combined with other feed additives in calf MR, as a potential strategy to further improve performance of the calves (Górka et al., 2021; 2023; Stefańska et al., 2021). Moreover, farmers often supplement other feed additives in MR that already contains probiotic bacteria (authors' observations); however, there is limited evidence demonstrating that combining various feed additives in calf MR results in additional benefits (Von Erhard et al., 2000; Stefańska et al., 2021). In fact, some combinations of feed additives in MR may negatively affect calf performance and health (Wood et al., 2019; Górka et al., 2023) or abolish the positive effect of feed additive already present in MR (Górka et al., 2023). Furthermore, the effect of such a practice can be highly variable depending on the farm where a combination of various feed additives is applied (Górka et al., 2023).

The effectiveness of feed additives may be also affected by the age of calves and their nutrition before the initiation of supplementation. Most studies investigating the effect of various feed additives in calf MR focused on the period from first 2 to 4 days of the calves' life (Von Erhard et al., 2000; Stefańska et al., 2021; Jahani-Azizabadi et al., 2022). However, feed additives can also be introduced in the diet later in the life of calves, especially when MR feeding begins after 7 to 14 days, following the administration of surplus colostrum or transition milk for an extended period of time (Górka et al., 2021; 2023). The feeding of colostrum or transition milk during the first days of calves' life can significantly impact the efficacy of feed additive supplementation. Prolonged colostrum feeding has known effects on the development of the calves' GIT and immune system development, and thus their growth and health (Van Soest et al., 2022). Among the many factors influencing calf body development, colostrum intake was also shown to affect the development of the intestinal microbiota (Fischer et al., 2018).

The aim of the study was to determine the effect of supplementing calf milk replacer containing probiotic bacteria with sodium butyrate, phytogenic compounds and egg yolk antibodies on selected bacteria counts in faeces. The study focused on calves that were fed surplus colostrum and transition milk for the initial 9 days of their lives. The hypothesis was that the combination of various feed additives with probiotic bacteria in MR might not necessarily have a beneficial effect on the intestinal microbiota of calves fed colostrum and transition milk for an extended period of time.

Material and methods

The study was carried out on a dairy farm located in the northwestern Poland (Gospodarstwo Rolno-Hodowlane Żydowo Sp. z o. o., Żydowo; Farm B). The experimental procedures were in accordance with Polish legislation, which is in line with EU Directive 2010/63/EU concerning the protection of animals used for scientific purposes.

A detailed description of the experimental design, housing and feeding of calves can be found in a companion paper (Górka et al., 2023), specifically in the description of Study 1B. Briefly, 96 Holstein calves (48 females and 48 males; average body weight of 45.3 ± 4.1 kg and a total serum protein level of 5.71 ± 0.73 g/l) were blocked by date of birth and sex at 10 days of age and then allocated to one of four treatments within each block: 1) MR containing probiotic bacteria but no other feed additives (control treatment; CTRL); 2) MR with sodium butyrate (SB; 3.4 kg/t, resulting in an intake of 3.1 g/calf/day; Adimix Easy, Nutriad, Belgium); 3) MR with phytogenic compounds (PC; 0.5 kg/t, resulting in an intake of 0.45 g/calf/day; Digestarom, Biomin, Austria) consisting mainly of caraway, liquorice extract, oak bark and vanilla flavour; and 4) MR with egg yolk antibodies (EY; egg yolk powder containing specific antibodies; 3 kg/tonne, resulting in an intake of 2.7 g/calf/day; Globigen Life Start, EW Nutrition, Germany). The MR (21% crude protein and 18% fat) used in the study contained *Bacillus licheniformis* and *B. subtilis* (1.3×10^6 CFU/g), and *Enterococcus faecium* (1.2×10^6 CFU/g). The addition of each feed additive to the MR followed the recommendations provided by the respective manufacturers. However, it is important to note that the manufacturers were not directly consulted, and their representatives were not involved in the preparation of study protocols.

Prior to the study, the calves were separated from their dams immediately after birth. The calves were then individually housed in straw-bedded hutches, which measured $150 \times 120 \times 125$ cm (length $\times$ width $\times$ high) and included an additional outside area of 150×120 cm. Within the first two hours of life, each calf received 4 litres of maternal colostrum via a stomach tube. Thereafter, a mixture of surplus colostrum, transition milk, and if necessary to obtain sufficient volume, whole milk (tank milk) was offered (3×2 l) until day 10 of life. From that day onwards, the calves were fed 6 l of MR three times a day (2 l/feeding; 150 g of MR powder in 1 l of water). The MR (Polmass Red Full, Polmass S.A., Bydgoszcz, Poland) contained 21% crude protein and 18% fat and was fed from a nipple bucket. Starting from the first day of the study, the calves had also free access to a texturised starter feed (Chrupka, Lira Wytwórnia Pasz, Krzywiń, Poland). The calves remained in the study until 60 days of age, thus the study lasted 50 days (from 10 to 60 days of age). Prior to the enrolment to the study, the intake of colostrum, transition milk, and whole milk by the calves during the first 9 days of life was not monitored.

Calves were weighed on the first day of the study and every 10 days thereafter. Milk replacer intake was monitored daily and the faecal score was assessed also daily using the scoring system proposed by Larson et al. (1977). Starter intake was determined by recording the amount of starter fed throughout the study period, and feed efficiency was calculated by dividing body weight gain by dry matter intake (Górka et al., 2023).

On days 14 ($\pm$ 2 days) and 28 ($\pm$ 2 days) of the study, faecal samples were collected from 8 randomly selected calves/treatment (4 females and 4 males) and analysed for the total number of bacteria, *Lactobacillus* spp., *Bifidobacterium* spp., *E.* coli, and *Clostridium perfringens* counts. The deviation in faecal sampling days ($\pm$ 2 days) was due to the fact that faecal samples were collected two times a week (Tuesday and Friday) and the number of samples collected each day had to be limited due to laboratory logistics. Therefore, the actual days of faecal sample collection were days 14.0 and 27.8 of the study.

The procedure of faecal sampling and bacterial analysis can be found elsewhere (Górka et al., 2021). Briefly, faecal samples were collected by manually stimulating the rectum of the calves, and collecting faeces into sterile containers (120 ml). For analysis of anaerobic bacterial counts, the containers were also placed in a foil bag with AnaeroGen Compact (Thermo Fisher Scientific Oxoid Ltd., Basingstoke, UK). Subsequently, the samples were transported to the laboratory of the Department of Biology and Animal Environment at the Bydgoszcz University of Science and Technology in a portable refrigerator. Ten grams of fresh faecal sample was diluted with 90 ml of 1% peptone water with 0.85% salt (Thermo Fisher Scientific Oxoid Ltd. Basingstoke, UK) and shaken for 3 min using a laboratory blender (BagMixer® 400CC, Interscience, Saint Nom la Brétèche, France). Subsequently, serial dilutions were made in saline peptone water and cultured onto selective media. Tryptic Soy Agar (TSA; Merck, Darmstadt, Germany) was used to determine the total number of bacteria. The obtained inoculations were incubated for 24 h at a temperature of 37 °C. Chromogenic Tryptone Bile X-glucuronide (TBX-Agar; Merck, Darmstadt, Germany) medium was used for the determination of β-glucuronidase-positive *E. coli*. The plates were incubated for 24 h at 44 °C. *Lactobacillus* spp. was cultured on De Man, Rogosa and Sharpe (MRS) agar (Lactobacillus Agar; Merck, Darmstadt, Germany). MRS agar contains polysorbate, acetate and manganese in its composition, which act as growth-promoting agents for lactobacilli. Incubation was carried out at the

temperature of 30 °C for 72 h. The culture plates were placed in 2.5-l AnaeroJar (Thermo Fisher Scientific Oxoid Ltd., Basingstoke, UK) containers with Anaerogen (Oxoid) sachets to ensure appropriate anaerobic conditions. *Bifidobacterium* spp. was cultured on Bifidus Selective Medium (BSM-Agar; Sigma-Aldrich, St. Louis, MO, USA). Incubation was carried out at a temperature of 37 °C for 36–48 h in anaerobic conditions. *C. perfringens* was grown on medium Tryptose Sulphite Cycloserine (TSC-Agar; Merck, Darmstadt, Germany). Incubation was carried out at a temperature of 37 °C for 36–48 h in anaerobic conditions. As a result of the reaction of sodium disulphite with iron (III) ammonium citrate, *C. perfringens* forms black colonies on medium surface, and the addition of cycloserine helps to inhibit the growth of accompanying bacteria. Bacterial species identification was performed on the basis of characteristic morphology of the colonies, Gram staining, and the biochemical analytical profile index tests API 20 A and API 20 E (BioMérieux Polska Sp z.o.o., Warsaw, Poland). The analysis was performed in triplicate. Only plates with colony-forming units (CFU) ranging from 15 to 300 were used for calculations.

The results are expressed in CFU/g fresh faeces and calculated as:

$$L = \frac{C}{[n_1 + (0.1 \times n_2)] \times d},$$

where: L – total number of bacteria (CFU/g of faeces); C – sum of all colonies grown on plates used for calculation; n_1 – number of plates from the first calculated dilution; n_2 – number of plates from the next calculated dilution; d – dilution index corresponding to the first calculated dilution.

Data were analysed using the MIXED procedure of SAS software (ver. 9.4, SAS Institute, Inc.). Faecal bacteria count data were log-transformed before the analysis and the results were analysed separately for each sampling point. Prior to data analysis, data normality and homogeneity of variance were tested using PROC UNIVARIATE of SAS. The statistical model included the fixed effects of treatment, sex of the calves, and the interaction between sex and treatment. The age of the calves on the day of sampling was initially included in the model as a covariate to account for initial age variation. However, it was found to be non-significant ($P > 0.10$) for all analysed parameters and was removed from the model. Furthermore, the passive immunity of calves was included as a covariate in the statistical model. Since this effect tended to ($P \leq 0.10$) be significant for some analysed parameters, it was left in the model. The analysis of other variables such as body weight, feed intake, and feed efficiency was conducted according to the same statistical model described above. Initial body weight was included in the model as a covariate for analysis of final body weight and feed efficiency. Faecal score data were analysed using PROC GLIMMIX of SAS and Poisson distribution. The statistical model included the effect of time (day of the study). When a significant interaction effect of treatment and sex of the calves was observed, means were separated using a Tukey adjustment in SAS. Significance was declared when $P \leq 0.05$, and trends were declared at $0.05 < P \leq 0.10$.

Results

Passive immune status (total serum protein concentration), initial and final body weight, MR and starter intake, feed efficiency, and faecal score of calves selected for faecal sampling are presented in Table 1. Total serum protein, initial body weight, MR intake, starter intake and the faecal score did not differ between treatments ($P \geq 0.18$), while final body weight tended to or was significantly higher in SB and EY calves compared to CTRL calves ($P \leq 0.10$). Furthermore, feed efficiency was significantly higher for PC and EY calves compared to CTRL calves ($P \leq 0.05$). However, it is important to interpret these differences with caution as they are based on a sample size of 8 calves/treatment and are not consistent with the results presented in the companion paper, which presents the effect of investigated factors on growth performance of calves on a larger number of animals (Górka et al., 2023). Regarding the interaction between effect of treatment and sex of the calves, only a few interactions were observed. Specifically, total bacteria counts on day 14 of the study tended to ($P = 0.06$) be affected by the interaction between effect of treatment and sex of the calves. The highest counts were observed for PC and EY bull calves, while the lowest for SB heifers. The total bacteria count on day 28 of the study was also affected by the interaction between effect of treatment and sex of the calves ($P = 0.05$), with the highest counts observed for EY heifers and the lowest for PC heifers. Moreover, *C. perfringens* counts were affected by the interaction between treatment and sex of the calves ($P = 0.05$), with the highest counts recorded for EY heifer calves and the lowest for PC heifers calves. However, the Tukey post-hoc mean separation did not detect differences between

Table 1. Passive immune status at the start of the study (serum total protein concentration), body weight, feed intake, feed efficiency, and faecal score of calves

	Group				SE	Contrasts[1]		
	CTRL	SB	PC	EY		1	2	3
N	8	8	8	8				
Total serum protein, g/l	5.88	5.83	5.69	5.51	0.327	0.93	0.69	0.44
Initial body weight, kg	45.0	42.9	45.5	45.1	1.35	0.30	0.80	0.97
Final body weight, kg	84.2	87.5	86.3	88.7	1.32	0.10	0.26	0.03
Milk replacer intake, kg DM[2]	43.9	44.1	44.1	44.1	0.06	0.25	0.18	0.19
Starter intake, kg DM	13.9	14.1	11.5	13.6	1.32	0.94	0.21	0.85
Feed efficiency, g gain/kg DM	684	737	750	768	23.1	0.13	0.05	0.02
Faecal score[3]	1.07	1.03	1.04	1.08	0.031	0.43	0.49	0.75

CTRL – animals fed a milk replacer with probiotic bacteria only, SB – animals fed a milk replacer containing probiotic bacteria and sodium butyrate (3.4 kg/t, 3.1 g/day/calf; Adimix Easy, Nutriad, Belgium), PC – animals fed a milk replacer containing probiotic bacteria and phytogenic compounds (0.5 kg/t, 0.45 g/day/calf; Digestarom, Biomin, Austria), EY – animals fed a milk replacer containing probiotic bacteria and egg yolk antibodies (egg yolk powder containing specific antibodies; 3 kg/t, 2.7 g/day/calf; Globigen Life Start, EW Nutrition, Germany), SE – standard error, DM – dry matter; [1] 1 – CTRL vs. SB, 2 – CTRL vs. PC, 3 – CTRL vs. EY; [2] cumulative intake throughout the study; [3] tendency to significant time effect ($P = 0.09$); $P < 0.05$ indicates significant differences

treatments for any of the listed parameters. Thus, the results concerning sex within each treatment are not presented and discussed further in the manuscript. This decision is additionally justified by the relatively small sample size (4 bulls and 4 heifers/treatment), which does not allow to accurately determine (i.e. sufficiently limit the statistical error) significant interactions between these effects.

On day 14 of the study, total bacteria counts were higher for EY calves compared to CTRL calves

($P = 0.02$; Table 2). Furthermore, *Lactobacillus* spp. counts were higher for PC and EY calves compared to CTRL calves ($P < 0.01$), whereas *Bifidobacterium* spp. counts were higher for SB, PC and EY calves compared to CTRL calves ($P \leq 0.02$). On the other hand, *E. coli* counts were higher for EY calves ($P = 0.02$) and tended to ($P = 0.06$) be higher for SB calves compared to CTRL. In consequence, the *Lactobacillus*-to-*Escherichia* ratio tended to ($P = 0.09$) be lower for SB compared to CTRL

Table 2. Bacterial counts in faeces on day 14 and 28 of the study ($\log_{10}$ CFU/g of fresh faeces)

	Group				SE	Contrasts[1]		
	CTRL	SB	PC	EY		1	2	3
N	8	8	8	8				
Day 14 of the study								
total bacteria	9.31	9.35	9.80	10.14	0.239	0.89	0.16	0.02
Lactobacillus spp.	7.38	7.37	8.12	8.22	0.125	0.95	<0.01	<0.01
Bifidobacterium spp.	7.26	7.78	8.33	8.37	0.148	0.02	<0.01	<0.01
E. coli	6.28	6.78	6.29	6.92	0.177	0.06	0.98	0.02
C. perfringens	3.88	3.76	3.70	3.82	0.138	0.55	0.34	0.75
Lactobacillus: Escherichia	1.18	1.09	1.30	1.20	0.036	0.09	0.03	0.77
Bifidobacterium: Escherichia	1.17	1.15	1.26	1.21	0.036	0.76	0.08	0.25
Dday 28 of the study								
total bacteria	9.48	8.99	9.04	9.36	0.192	0.09	0.13	0.68
Lactobacillus spp.	7.01	7.24	7.23	7.56	0.148	0.28	0.29	0.02
Bifidobacterium spp.	7.06	7.44	7.35	6.84	0.189	0.18	0.30	0.40
E. coli	6.23	6.44	6.07	6.55	0.199	0.45	0.59	0.26
C. perfringens	3.87	3.58	3.60	2.97	0.140	0.16	0.20	<0.01
Lactobacillus: Escherichia	1.14	1.14	1.20	1.16	0.046	0.99	0.36	0.74
Bifidobacterium: Escherichia	1.14	1.17	1.22	1.04	0.044	0.67	0.24	0.14

CTRL – animals fed a milk replacer with probiotic bacteria only, SB – animals fed a milk replacer containing probiotic bacteria and sodium butyrate (3.4 kg/t, 3.1 g/day/calf; Adimix Easy, Nutriad, Belgium), PC – animals fed a milk replacer containing probiotic bacteria and phytogenic compounds (0.5 kg/t, 0.45 g/day/calf; Digestarom, Biomin, Austria), EY – animals fed a milk replacer containing probiotic bacteria and egg yolk antibodies (egg yolk powder containing specific antibodies; 3 kg/t, 2.7 g/day/calf; Globigen Life Start, EW Nutrition, Germany), SE – standard error, CFU – colony forming unit; [1] 1 – CTRL vs. SB, 2 – CTRL vs. PC, 3 – CTRL vs. EY; $P < 0.05$ indicates significant differences

calves, but was higher ($P = 0.03$) for PC calves compared to CTRL calves. The ratio of *Bifidobacterium*-to-*Escherichia* also tended to ($P = 0.08$) be higher for PC compared to CTRL.

On day 28 of the study, total bacteria counts tended to ($P = 0.09$) be lower for SB calves compared to CTRL calves. *Lactobacillus* spp. counts were higher ($P = 0.02$), whereas that of *C. perfringens* counts were lower ($P < 0.01$) for EY calves compared to CTRL calves.

Additionally, several differences between bull and heifer calves were detected. On day 14 of the study, bull calves had higher *Lactobacillus* spp. counts (7.96 vs. 7.59; $P < 0.01$) but lower *E. coli* counts (6.38 vs. 6.75; $P = 0.05$) compared to heifer calves. Consequently, the *Lactobacillus*-to-*Escherichia* ratio was higher for bull calves compared to heifer calves (1.27 vs. 1.13; $P < 0.01$). Moreover, the ratio of *Bifidobacterium*-to-*Escherichia* was higher for bulls compared to heifers on day 14 of the study (1.24 vs. 1.17; $P = 0.03$). However, as the study did not primarily focus on sex differences, these findings are not presented in detail and will only be briefly discussed in the later parts of the manuscript.

Discussion

Before discussing the result in more detail, it should be mentioned that faecal bacteria counts were used in the current study as an indicator of the effect of the investigated feed additives on intestinal bacteria. It should be noted that there can be differences between faecal bacteria counts and actual intestinal colonisation (Malmuthuge et al., 2015). Nevertheless, the presence and counts of specific bacteria such as *E. coli* or *C. perfringens* in faeces can be used as markers of GIT colonisation by these undesirable bacteria, whereas the *Lactobacillus*-to-*Escherichia* ratio can serve as an indicator of intestinal microbiome dysbiosis (Kehoe et al., 2008; Malmuthuge et al., 2015; Górka et al., 2021). Furthermore, it is important to mention once again that the current study utilised a relatively small sample size of 8 calves per treatment. Therefore, caution should be made when interpreting the results pertaining to the growth performance of the calves. The findings presented in this work are part of a larger project (Górka et al., 2023) that investigated the impact of SB, PC, and EY supplementation in MR on the growth performance of calves in a series of studies involving a larger number of animals. Consequently, when appropriate, references to the companion paper will be made, and the growth performance of calves described and dis-

cussed briefly in order to provide better context for interpreting the results of faecal bacteria presented in the current study.

Among the investigated feed additives, SB supplementation in MR has a well-established positive impact on GIT development and function in calves (Górka et al., 2011). Moreover, the dietary supplementation of SB has shown positive effects on intestinal microbiota in calves. For example, the colonisation of *Mogibacterium*, which can adversely affect GIT health, was reduced in the colon of calves fed MR with SB (O'Hara et al., 2018). This effect was accompanied by a tendency towards higher average daily gain (ADG) and feed efficiency of calves. Dietary SB supplementation was also shown to reduce GIT colonisation by *E. coli* in piglets (Xiong et al., 2016). However, it should be noted that not all studies have consistently observed the beneficial effects of SB supplementation in calf MR. Wolfswinkel (2017) reported an increased incidence of diarrhoea in calves fed MR with SB. In the study of Wood et al. (2019), SB supplementation in MR was associated with a higher risk of calf mortality, but it was supplemented with *B. subtilis*, as in the current study. Partly consistent with studies showing not only positive effects of SB supplementation in calf MR, in the current study SB supplementation resulted in higher *E. coli* counts in faeces and a lower *Lactobacillus*-to-*Escherichia* ratio on day 14 of the study, indicating a negative impact on the intestinal bacterial community. Furthermore, the growth performance of the calves was negatively affected, as described in detail in the companion paper (Górka et al., 2023). Similar negative effects of SB supplementation have also been observed in broiler chickens, where it led to a reduction in *Lactobacillus* counts in the intestinal chyme (Hu and Guo, 2007). These findings suggest that SB supplementation in combination with probiotic bacteria, as in the case of *B. subtilis* and *E. faecium* in this study, may not always yield positive results. It is possible that the combination of SB with probiotic bacteria overstimulates the immune system, as suggested by Wood et al. (2019), leading to a negative impact on GIT function and calf performance.

Numerous studies have demonstrated the positive impact of PC on growth performance and health issues prevention in calves (Froehlich et al., 2017; Stefańska et al., 2021; Jahani-Azizabadi et al., 2022). When supplemented in feed, PC action is not limited to their antimicrobial effects, which are particularly desired in newborn calves that are susceptible to various infections. PC are also known to affect digestive enzyme secretion and exhibit antioxidant and

anti-inflammatory properties (Froehlich et al., 2017; Upadhaya and Kim, 2017). Importantly, a combination of phytogenic feed additive with probiotic bacteria in MR was shown to have an synergistic impact on the growth performance of calves (Stefańska et al., 2021). Among the investigated feed additives, the combination of probiotics and PC had the most positive influence on calf performance. It increased feed efficiency and decreased the likelihood of diarrhoea in calves, as described in detail in the companion paper (Górka et al., 2023). These benefits of PC supplementation in MR were accompanied by higher *Lactobacillus* spp. and *Bifidobacterium* spp. counts in faeces on day 14 of the study, and consequently higher *Lactobacillus*-to-*Escherichia* and *Bifidobacterium*-to-*Escherichia* ratios. Thus, the positive effect of PC supplementation in the current study can be at least partly attributed to their impact on the development of gastrointestinal microbiota. In other studies, supplementation with herbal extracts or essential oils also reduced the occurrence or intensity of diarrhoea in calves (Froehlich et al., 2017; Jahani-Azizabadi et al., 2022). In addition, essential oil supplementation reduced the counts of *E. coli* in the rectum in piglets (Zeng et al., 2015).

These findings collectively support the notion that PC supplementation in calf MR can yield positive outcomes, even when combined with probiotic bacteria and when calves are fed colostrum and transition milk for an extended period of time. Further studies are needed to fully understand the mechanisms behind these positive effects and to elucidate the specific pathways through which PC supplementation influences the gut microbiota and promotes calf health.

Supplementing EY in MR exerted a similar effect on faecal bacteria on day 14 of the study as with PC supplementation. Both *Lactobacillus* spp. and *Bifidobacterium* spp. counts in faeces were increased; however, simultaneously *E. coli* counts were increased. Moreover, the ADG of calves was reduced in the first 20 days of the study when EY were supplemented in MR (see companion paper of Górka et al. (2023)), which is difficult to explain. EY supplementation in MR aims to bind and 'neutralise' pathogens that enter GIT and can adversely affect its function (Diraviyam et al., 2014). However, based on a meta-analysis of available studies, EY supplementation did not consistently reduce GIT-related diseases in newborn animals, as some studies reported no effect of EY addition in feeds (Diraviyam et al., 2014). Considering that the calves in the current study were fed colostrum and transition

milk for the first 9 days of life, which indisputably affected the development of the intestinal bacteria community (Fischer et al., 2018), it is possible that the supplementation of EY had both positive (increased *Lactobacillus* spp. and *Bifidobacterium* spp. counts in faeces) and negative effects on this community shortly after calves were transitioned to MR feeding. However, it should be noted that in piglets, EY supplementation directly after weaning was shown to prevent GIT colonisation by *E. coli* (Marquardt et al., 1999). Similarly, in calves, dietary EY supplementation was consistently demonstrated to prevent diarrhoea of various aetiology (Özpinar et al., 1996; Ikemori et al., 1997). Alternatively, a negative interaction between probiotic bacteria present in the experimental MR and EY can be considered. However, a study by Von Erhard et al. (2000) reported a synergistic impact on the growth parameters of calves when a combination of probiotic bacteria and EY was added to MR. On the other hand, EY supplementation increased *Lactobacillus* spp. counts and decreased *C. perfringens* counts in faeces on day 28 of the current study, which was accompanied by an increase in ADG of calves (Górka et al., 2023). Therefore, when considering prophylactic measures for the entire rearing period, the incorporation of EY into MR may be beneficial, also for calves initially fed colostrum and transition milk during the first several days of life.

At least several studies have shown differences in ADG and feed intake between bull and heifer calves (Greenwood et al., 1997; Hohmann et al., 2021). These differences can be attributed to physiological differences between males and females. The results of the current study show that faecal bacteria counts may differ between bull and heifer calves, with higher counts of *Lactobacillus* spp. and *Bifidobacterium* spp. expected in bull calves. This may partially explain the reported differences between bull and heifer calves in terms of the probability of GIT-related diseases (Al Mawly et al., 2015). Future studies focusing on the specific differences in the development of intestinal bacteria between bull and heifer calves would contribute to a better understanding of their unique nutritional requirements and help optimizing feed additive usage in calf MR.

Conclusions

Of the investigated feed additives, PC supplementation had the most positive impact on faecal bacteria composition in calves fed MR containing probiotic bacteria, as well as colostrum

and transition milk in the first 9 days of life. This was indicated by increased counts of *Lactobacillus* spp. and *Bifidobacterium* spp. and higher ratios of *Lactobacillus*-to-*Escherichia* and *Bifidobacterium*-to-*Escherichia* in faeces. In contrast, SB supplementation showed less favourable effects on faecal bacteria counts. It tended to increase *E. coli* counts in faeces and lower the *Lactobacillus*-to-*Escherichia* ratio, which indicated a rather negative impact on the investigated faecal bacteria composition in calves. EY supplementation was found to reduce *C. perfringens* counts in faeces, suggesting potential benefits of this supplement.

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Conflict of interest

The Authors declare that there is no conflict of interest.

References

Al Mawly J., Grinberg A., Prattley D., Moffat J., Marshall J., French N., 2015. Risk factors for neonatal calf diarrhoea and enteropathogen shedding in New Zealand dairy farms. Wet. J. 203, 155–160, https://doi.org/10.1016/j.tvjl.2015.01.010

Brand T., Hünerberg M., McAllister T.A., He M., Saleem A.M., Shen Y., Miller B., Yang W. 2019. Impact of a phytogenic feed additive on growth performance, feed intake, and carcass traits of finishing steers. Transl. Anim. Sci. 3, 1162–1172, https://doi.org/10.1093/tas/txz109

Cangiano L.R., Yohe T.T., Steele M.A., Renaud D.L., 2020. Invited review: Strategic use of microbial-based probiotics and prebiotics in dairy calf rearing. Appl. Anim. Sci. 36, 630–651, https://doi.org/10.15232/aas.2020-02049

Cook S.R., Bach S.J., Stevenson S.M.L., DeVinney R., Frohlich A.A., Fang L., McAllister T.A., 2005. Orally administered anti-*Escherichia coli* O157:H7 chicken egg yolk antibodies reduce fecal shedding of the pathogen by ruminants. Can. J. Anim. Sci. 85, 291–299, https://doi.org/10.4141/a04-076

Diraviyam T., Zhao B., Wang Y., Schade R., Michael A., Zhang X., 2014. Effect of chicken egg yolk antibodies (IgY) against diarrhea in domesticated animals: a systematic review and meta-analysis. PLoS One 9, e97716, https://doi.org/10.1371/journal.pone.0097716

Fischer A.J., Song Y., He Z., Haines D.M., Guan L.L., Steele M.A., 2018. Effect of delaying colostrum feeding on passive transfer and intestinal bacterial colonization in neonatal male Holstein calves. J. Dairy Sci. 101, 3099–3109, https://doi.org/10.3168/jds.2017-13397

Frieten D., Gerbert C., Koch C., Dusel G., Eder K., Kanitz E., Weitzel J.M., Hammon H.M., 2017. Ad libitum milk replacer feeding, but not butyrate supplementation, affects growth performance as well as metabolic and endocrine traits in Holstein calves. J. Dairy Sci. 100, 6648–6661, https://doi.org/10.3168/jds.2017-12722

Froehlich K.A., Abdelsalam K.W., Chase C., Koppien-Fox J., Casper D.P., 2017. Evaluation of essential oils and prebiotics for newborn dairy calves. J. Anim. Sci. 95, 3772–3782, https://doi.org/10.2527/jas.2017.1601

Górka P., Budzińska K., Budziński W., Jankowiak T., Kehoe S., Kański J., 2021. Effect of probiotic and nucleotide supplementation in milk replacer on growth performance and fecal bacteria in calves. Livest. Sci. 250, 104556, https://doi.org/10.1016/j.livsci.2021.104556

Górka P., Kowalski Z.M., Pietrzak P., Kotunia A., Jagusiak W., Zabielski R., 2011. Is rumen development in newborn calves affected by different liquid feeds and small intestine development? J. Dairy Sci. 94, 3002–3013, https://doi.org/10.3168/jds.2010-3499

Górka P., Milik J., Budziński W., Przybyło M., Jankowiak T., Budzińska K., 2023. Effect of sodium butyrate, phytogenic compounds and egg yolk antibodies supplementation in calf milk replacer containing probiotic bacteria on farms feeding a mixture of surplus colostrum and transition milk to calves in their first days of life. Anim. Feed Sci. Technol. 302, 115675, https://doi.org/10.1016/j.anifeedsci.2023.115675

Greenwood R.H., Morrill J.L., Titgemeyer E.C., 1997. Using dry feed intake as a percentage of initial body weight as a weaning criterion. J. Dairy Sci. 80, 2542–2546, https://doi.org/10.3168/jds.S0022-0302(97)76208-8

Hohmann L.G., Yin T., Schweizer H., Giambra I.J., Konig S., Scholz A.M., 2021. Comparative effects of milk containing A1 versus A2 beta-casein on health, growth and beta-casomorphin-7 level in plasma of neonatal dairy calves. Animals 11, 55, https://doi.org/10.3390/ani11010055

Hu Z., Guo Y., 2007. Effects of dietary sodium butyrate supplementation on the intestinal morphological structure, absorptive function and gut flora in chickens. Anim. Feed Sci. Technol. 132, 240–249, https://doi.org/10.1016/j.anifeedsci.2006.03.017

Ikemori I., Ohta M., Umeda K., Icatlo F.C., Kuroki M., Yokoyama H., Kodama Y., 1997. Passive protection of neonatal calves against bovine coronavirus-induced diarrhea by administration of egg yolk or colostrum antibody powder. Vet. Microbiol. 58, 105–111, https://doi.org/10.1016/S0378-1135(97)00144-2

Jahani-Azizabadi H., Baraz H., Bagheri N., Ghaffari M.H., 2022. Effects of a mixture of phytobiotic-rich herbal extracts on growth performance, blood metabolites, rumen fermentation, and bacterial population of dairy calves. J. Dairy Sci. 105, 5062–5073, https://doi.org/10.3168/jds.2021-20687

Kehoe S.I., Heinrichs A.J., Baumrucker C.R., Greger D.L., 2008. Effects of nucleotide supplementation in milk replacer on small intestinal absorptive capacity in dairy calves. J. Dairy Sci. 91, 2759–2770, https://doi.org/10.3168/jds.2007-0751

Kholif A.E., Hassan A.A., El Ashry G.M., Bakr M.H., El-Zaiat H.M., Olafadehan O.A., Matloup O.H., Sallam S.M.A., 2021. Phytogenic feed additives mixture enhances the lactational performance, feed utilization and ruminal fermentation of Friesian cows. Anim. Biotechnol. 32, 708–718, https://doi.org/10.1080/10495398.2020.1746322

Larson L.L., Owen F.G., Albright J.L., Appleman R.D., Lamb R.C., Muller L.D., 1977. Guidelines toward more uniformity in measuring and reporting calf experimental data. J. Dairy Sci. 60, 989–991, https://doi.org/10.3168/jds.S0022-0302(77)83975-1

Malmuthuge N., Griebel P.J., Guan L.L., 2015. The gut microbiome and its potential role in the development and function of newborn calf gastrointestinal tract. Front. Vet. Sci. 2, 36, https://doi.org/10.3389/fvets.2015.00036

Marquardt R.R., Jin L.Z., Kim J.-W., Fang L., Frohlich A.A., Baidoo S.K., 1999. Passive protective effect of egg-yolk antibodies against enterotoxigenic *Escherichia coli* K88[+] infection in neonatal and early-weaned piglets. FEMS Immunol. Med. Microbiol. 23, 283–288, https://doi.org/10.1111/j.1574-695X.1999.tb01249.x

O'Hara E., Kelly A., McCabe M.S., Kenny D.A., Guan L.L., Waters S.M., 2018. Effect of a butyrate-fortified milk replacer on gastrointestinal microbiota and products of fermentation in artificially reared dairy calves at weaning. Sci. Rep. 8, 14901, https://doi.org/10.1038/s41598-018-33122-6

Özpinar H., Erhard M.H., Aytug N., Özpinar A., Baklaci C., Karamüptüoglu S., Hofmann A., Lösch U., 1996. Dose-dependent effects of specific egg-yolk antibodies on diarrhea of newborn calves. Prev. Vet. Med. 27, 67–73, https://doi.org/10.1016/0167-5877(95)00561-7

Plaza-Diaz J., Ruiz-Ojeda F.J., Gil-Campos M., Gil A., 2019. Mechanisms of action of probiotics. Adv. Nutr. 10, S49–S66, https://doi.org/10.1093/advances/nmy063

Roodposhti P.M., Dabiri N., 2012. Effects of probiotic and prebiotic on average daily gain, fecal shedding of *Escherichia coli*, and immune system status in newborn female calves. Asian Australas. J. Anim. Sci. 25, 1255–1261, https://doi.org/10.5713/ajas.2011.11312

Signorini M.L., Soto L.P., Zbrun M.V., Sequeira G.J., Rosmini M.R., Frizzo L.S., 2012. Impact of probiotic administration on the health and fecal microbiota of young calves: a meta-analysis of randomized controlled trials of lactic acid bacteria. Res. Vet. Sci. 93, 250–258, https://doi.org/10.1016/j.rvsc.2011.05.001

Stefańska B., Sroka J., Katzer F., Goliński P., Nowak W., 2021. The effect of probiotics, phytobiotics and their combination as feed additives in the diet of dairy calves on performance, rumen fermentation and blood metabolites during the preweaning period. Anim. Feed Sci. Technol. 272, 114738, https://doi.org/10.1016/j.anifeedsci.2020.114738

Upadhaya S.D., Kim I.H., 2017. Efficacy of phytogenic feed additive on performance, production and health status of monogastric animals – a review. Ann. Anim. Sci. 17, 929–948, https://doi.org/10.1515/aoas-2016-0079

Van Soest B., Weber Nielsen M., Moeser A.J., Abuelo A., VandeHaar M.J., 2022. Transition milk stimulates intestinal development of neonatal Holstein calves. J. Dairy Sci. 105, 7011–7022, https://doi.org/10.3168/jds.2021-21723

Von Erhard M.H., Leuzinger K., Stangassinger M., 2000. Studies on the prophylactic effect of feeding probiotics, pathogen-specific colostrum antibodies or egg yolk antibodies in newborn calves. J. Anim. Physiol. Anim. Nutr. 84, 85–94, https://doi.org/10.1046/j.1439-0396.2000.00284.x

Wolfswinkel T.L., 2017. The effects of feeding prebiotics, antibiotics, and alternative proteins during the preweaning period to dairy calves on growth, health, and the gastrointestinal microbiota, PhD. Iowa State University. Ames, IA (USA), https://doi.org/10.31274/etd-180810-5082

Wood D.R., Blome R.M., Keunen A.J., Keunen B.W., Crenshaw J.D., Campbell J.M., Renaud D.L., 2019. Short communication: effects of porcine plasma or combined sodium butyrate and *Bacillus subtilis* on growth and health of grain-fed veal calves. J. Dairy Sci. 102, 7183–7188, https://doi.org/10.3168/jds.2019-16672

Xiong H., Guo B., Gan Z., Song D., Lu Z., Yi H., Wu Y., Wang Y., Du H.S., 2016. Butyrate upregulates endogenous host defense peptides to enhance disease resistance in piglets via histone deacetylase inhibition. Sci. Rep. 6, 27070, https://doi.org/10.1038/srep27070

Zeng Z., Xu X., Zhang Q., Li P., Zhao P., Li Q., Liu J., Piao X.S., 2015. Effects of essential oil supplementation of a low-energy diet on performance, intestinal morphology and microflora, immune properties and antioxidant activities in weaned pigs. Anim. Sci. J. 86, 279–285, https://doi.org/10.1111/asj.12277