

Different Methods of Eubiotic Feed Additive Provision Affects Health, Performance, Fermentation, and Metabolic Status of Dairy Calves During The Preweaning Period

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Abstract

Background

The aim of this study was to evaluate whether different methods of providing eubiotic feed additives, to neonatal calves during the preweaning period, can improve the health, performance, rumen fermentation, and metabolic status of the calves. Forty-four Holstein-Friesian dairy calves, were divided into one of four treatment groups for the 8 week trial. The eubiotic feed additives consisted of a combination of probiotic *Lactobacillus* spp. (multiple-strains) at a dose of 250 mg/calf/d and phytobiotics containing rosmarinic acid, as the main bioactive compound, at a dose of 50 mg/calf/d. Treatment differed by the methods that the eubiotic feed additives were provided, and the groups were as follows: CON (control, without eubiotic feed additive in their milk replacer or their starter feed), MR (eubiotic feed additives added to the milk replacer), SF (eubiotic feed additives added to the starter feed), MRS (eubiotic feed additives added to the milk replacer and the starter feed). Individual intake of starter feed was measured daily and body weights weekly until 56 d of age.

Results

The body weight of the MR treatment group calves were higher on days 28 and 56. Including the eubiotic feed additive in the milk replacer affected increasing average daily gain, starter intake and total dry matter intake from d 29 to d 56 and the overall experimental period in comparison to the CON group. The calves of the MR treatment had lower fecal scores from d 3 to d 28, number of parasite oocysts/cysts per gram of feces on d 28, and the occurrences of faecal consistency scores of 3 (mild diarrhea) and 4 (confirmation diarrhea) were 3.2 and 3.0 times lower, respectively, compared with animals of the CON group. Also, in this group higher ruminal concentrations of total volatile fatty acids, propionate, and butyrate were noted on d 56 compared to CON group. Adding eubiotics into milk replacer resulted the highest concentrations of blood insulin-like growth factor-I and β -hydroxybutyrate from d 29 to 56 and through the entire experimental period.

Conclusion

The addition of eubiotic feed additives into the milk replacer can improve health, performance, rumen fermentation, and biochemical blood indices in dairy calves during the preweaning period.

Background

Early-life nutrition has become a topic of increasing research interest because the development of well-growing dairy calves and heifers play an important role in the future economic success of all dairy farms, and therefore the growth phase occurring between birth and weaning is of major economic importance [1, 2]. Overall, the health status of the preweaned dairy calves can greatly affect lifelong production,

including growth, reproductive efficiency, and milk production [3]. Calves are at a greater risk of dying during the first 21 days of life [4]. The incidence for mortality in the perinatal period, defined as the duration from birth to 48 hours after birth, ranges in dairy herds worldwide between 3% and 9% [5]. In USA dairy herds, current mortality rates of 5% and morbidity rates of 34% were published for preweaning calves [6]. The UK Department of the Environment, Food and Rural Affairs reported that economic losses from calf mortality were around £60 million/year [7]. Also, disease during the neonatal stage, especially infectious diseases such as diarrhea, significantly affect the economic viability of dairy herds, due to the costs associated with calf mortality, treatments, long-term effects on performance, and loss of genetic potential for future herd improvement [8]. Feeding management during the neonatal and preweaning period has a great impact on the success of calf rearing and, in addition, affects health and performance in later life [2, 9]. Although mortality in calves is unlikely to be entirely eradicated, reducing it as much as possible should be a goal [10]. Therefore, understanding the relationship between management practices, nutrition strategies, and calf health is essential for minimizing morbidity and mortality and enhancing future production. To solve infection diseases associated with diarrhea and respiratory disease, in intensive rearing and management systems, diets have been supplemented widely with antibiotics, as feed additives. However, public and scientific concern about the use of antibiotics as feed additives in animal production (antibiotic resistance, environmental contamination, and foods of animal origin) lead the European Union (EU) to ban antibiotics used in livestock as production enhancers 1st January 2006 [11]. Therefore, new studies have been undertaken in recent years to develop alternatives to antibiotics such as natural feed additives for reducing morbidity and mortality in special-fed veal calves. Considerable evidence exists in the literature of the potential effects of a natural feed additives on the health, growth performance and rumen fermentation of dairy calves. The aims of most of the published research in dairy calf nutrition were to compare the effectiveness of the different types of feed additives such as probiotic [12, 13], prebiotics [14, 15], phytogetic substances [16], essential oils [17] or their blends, especially combinations of essential oils and prebiotics [18, 19]. Recent results showed that a eubiotic feed additive, consisting of a combination of a multi-strain probiotic (containing *Lactobacillus casei*, *Lactobacillus salivarius*, and *Lactobacillus sakei*) and herbal extracts with rosmarinic acid, as the main bioactive component, improved the health status (decreasing diarrhea occurrence, *Cryptosporidium* spp., and *Giardia duodenalis* prevalence), starter intake, total dry matter intake (TDMI), growth performance, and metabolic status of dairy calves during the preweaning period [20]. Feed additives can be mixed with liquid feeds, such as whole milk or milk replacer, or with solid feeds, such as the calf starter; however only very limited scientific data are available comparing the effects of administering the feed additives with all these dietary offering methods on calf health, growth performance, and metabolic status during the preweaning period [21]. This information would be particularly useful for preventing infectious diseases that cause diarrhea and modeling the effect on growth of dairy calves, especially during the important preweaning period. As beneficial effects of eubiotic feed additive were already described, this study aimed to evaluate whether the method of eubiotic feed additive provision, during the preweaning period to neonatal calves, can enhance calf health, performance, rumen fermentation, and metabolic status. We hypothesized that the method of eubiotic feed additive provision can influence

diarrhea occurrence, the growth performance, subsequent rumen environment and blood metabolites of dairy calves during the preweaning period.

Results

Feed intake and growth performance are presented in Table 1. The treatment groups differed in starter intake, TDMI, ADG and BW. As expected, calves consumed little solid feed during the first 4 wk of life. Calves consumed more starter intake and TDMI depending on treatment group during the 29–56 d period and the overall experimental period ($P \leq 0.05$), and the greatest effect was noted in the MR treatment group, where eubiotic was mixed in with the milk replacer. Greater BW at weaning and at the end of the experiment ($P \leq 0.05$) were noted in the MR treatment group compared to other experimental groups. Also, the calves fed milk replacer containing eubiotic (MR treatment) had higher ($P \leq 0.05$) ADG during 29–56 d and the overall experimental periods, respectively. In the current study, calves fed MR treatment had the lowest fecal scores from d 3 to d 28 ($P \leq 0.05$). The MR calves had lower number of parasite oocysts/cysts per gram of feces (EPG) on d 28 ($P \leq 0.05$) compared to the CON treatment group. Also, the numbers of score 3 indicating mild diarrhea and score 4 confirmation diarrhea occurrence were respectively 3.2 and 3.0 times lower ($P \leq 0.05$) for calves fed MR treatment compared with animals in the CON group, without feed additive during the entire experimental period (Table 2). Starter intake, TDMI, total CP intake, ADG, and biometric measurements such as changes in BL, HH, HW, HG increased with the age of calves (effect of the period; $P < 0.001$). No effects were detected based on the method of the eubiotic feed additive was provided on milk replacer intake, total CP intake, FE, and changes in all biometric measurements ($P > 0.05$).

Table 1

Different methods of eubiotic feed additive provision on intake and growth performance in dairy calves

Item	Treatment ¹				SEM	P-values		
	CON	MR	SF	MRS		Treatment	Period	Treatment x Period
Starter intake (kg/d)								
Period 3–28 d	0.26	0.27	0.22	0.28	0.11	0.46	< 0.001	0.10
Period 29–56 d	0.47 ^b	0.78 ^a	0.54 ^{ab}	0.59 ^{ab}	0.14	0.05	< 0.001	0.09
Overall 3–56 d	0.37 ^b	0.54 ^a	0.39 ^{ab}	0.44 ^{ab}	0.12	0.05	< 0.001	0.11
Milk replacer intake (kg/d)								
Period 3–28 d	0.81	0.81	0.81	0.81	0.01	0.62	0.59	0.84
Period 29–56 d	0.68	0.68	0.68	0.68	0.06	0.54	0.79	0.84
Overall 3–56 d	0.74	0.74	0.74	0.74	0.04	0.43	0.56	0.84
TDMI ² (kg/d)								
Period 3–28 d	1.07	1.08	1.03	1.09	0.06	0.42	< 0.001	0.24
Period 29–56 d	1.15 ^b	1.46 ^a	1.22 ^{ab}	1.27 ^{ab}	0.08	0.05	< 0.001	0.22
Overall 3–56 d	1.11 ^b	1.28 ^a	1.13 ^{ab}	1.18 ^{ab}	0.04	0.05	< 0.001	0.36
Total CP intake (kg/d)								
Period 3–28 d	0.26	0.26	0.25	0.27	0.01	0.32	< 0.001	0.32
Period 29–56 d	0.28	0.35	0.29	0.31	0.04	0.52	< 0.001	0.42

¹ *Treatment CON* (control: without eubiotic feed additive in their milk replacer or their starter feed: n = 11); *MR* (eubiotic feed additive added to milk replacer: n = 11); *SF* (eubiotic feed additive added to starter feed: n = 11); *MRS* (eubiotic feed additive added to milk replacer and starter feed: n = 11); ² *TDMI* total dry matter intake from milk replacer and starter feed (kg/d); ³ *ADG* average daily gain (kg/d) in period 3-28d = (((weaning BW - initial BW)/25 d); in period 29-56d = (((final BW - weaning BW)/28 d); in overall period = (((final BW - initial BW)/53 d)); ⁴ *FE* feed efficiency expressed as ADG (kg/d) to TDMI (kg/d) ratio; ⁵ *EPG* number of parasite oocysts/cysts per gram of feces; ^{a-b} Means within a column with different superscripts differ ($P \leq 0.05$).

Item	Treatment ¹				SEM	P-values		
	CON	MR	SF	MRS		Treatment	Period	Treatment x Period
Overall 3–56 d	0.27	0.31	0.27	0.29	0.02	0.35	< 0.001	0.35
Body weight (kg)								
Initial (3 d)	44.5	44.3	44.6	44.4	0.46	0.45	-	-
Weaning (28 d)	53.4 ^b	55.5 ^a	51.9 ^b	51.6 ^b	0.66	0.05	-	-
Final (56 d)	70.5 ^b	77.5 ^a	69.3 ^b	69.6 ^b	0.87	0.05	-	-
ADG ³ (kg/d)								
Period 3–28 d	0.36	0.45	0.29	0.29	0.15	0.93	< 0.001	0.97
Period 29–56 d	0.60 ^b	0.79 ^a	0.62 ^b	0.64 ^b	0.22	0.05	< 0.001	0.50
Overall 3–56 d	0.49 ^b	0.63 ^a	0.47 ^b	0.48 ^b	0.19	0.05	< 0.001	0.95
FE ⁴								
Period 3–28 d	0.34	0.46	0.38	0.37	0.10	0.32	0.46	0.10
Period 29–56 d	0.54	0.54	0.50	0.48	0.13	0.83	0.19	0.13
Overall 3–56 d	0.46	0.51	0.45	0.44	0.42	0.32	0.82	0.42
Body length change (cm)								
Period 3–28 d	5.10	5.90	5.90	5.80	1.10	0.19	< 0.001	0.28
Period 29–56 d	7.50	7.30	6.40	6.70	1.30	0.62	< 0.001	0.99
Overall 3–56 d	12.6	13.2	12.3	12.5	1.48	0.22	< 0.001	0.81

¹ Treatment CON (control: without eubiotic feed additive in their milk replacer or their starter feed: n = 11); MR (eubiotic feed additive added to milk replacer: n = 11); SF (eubiotic feed additive added to starter feed: n = 11); MRS (eubiotic feed additive added to milk replacer and starter feed: n = 11); ² TDMI total dry matter intake from milk replacer and starter feed (kg/d); ³ ADG average daily gain (kg/d) in period 3-28d = (((weaning BW - initial BW)/25 d); in period 29-56d = (((final BW - weaning BW)/28 d); in overall period = (((final BW - initial BW)/53 d); ⁴ FE feed efficiency expressed as ADG (kg/d) to TDMI (kg/d) ratio; ⁵ EPG number of parasite oocysts/cysts per gram of feces; ^{a-b} Means within a column with different superscripts differ ($P \leq 0.05$).

Item	Treatment ¹				SEM	P-values		
	CON	MR	SF	MRS		Treatment	Period	Treatment x Period
Hip height change (cm)								
Period 3–28 d	5.20	4.90	4.10	4.20	0.78	0.25	< 0.001	0.28
Period 29–56 d	5.40	6.00	5.50	4.50	0.84	0.34	< 0.001	0.99
Overall 3–56 d	10.6	10.9	9.60	8.70	0.87	0.11	< 0.001	0.81
Hip width change (cm)								
Period 3–28 d	2.20	1.80	2.2	3.10	0.29	0.38	< 0.001	0.28
Period 29–56 d	3.20	3.40	2.2	1.90	0.27	0.65	< 0.001	0.99
Overall 3–56 d	5.40	5.20	4.4	5.00	0.40	0.47	< 0.001	0.81
Heart girth change (cm)								
Period 3–28 d	6.00	4.10	5.20	6.20	0.59	0.24	< 0.001	0.28
Period 29–56 d	9.40	11.4	8.10	7.20	0.95	0.16	< 0.001	0.99
Overall 3–56 d	15.4	15.5	13.3	13.4	1.10	0.09	< 0.001	0.81
Fecal score								
Period 3–28 d	1.72 ^a	1.10 ^b	1.47 ^a	1.25 ^{ab}	0.02	0.05	< 0.001	0.034
Period 29–56 d	1.09	1.06	1.06	1.06	0.01	0.32	< 0.001	0.031

¹ *Treatment CON* (control: without eubiotic feed additive in their milk replacer or their starter feed: n = 11); *MR* (eubiotic feed additive added to milk replacer: n = 11); *SF* (eubiotic feed additive added to starter feed: n = 11); *MRS* (eubiotic feed additive added to milk replacer and starter feed: n = 11); ² *TDMI* total dry matter intake from milk replacer and starter feed (kg/d); ³ *ADG* average daily gain (kg/d) in period 3-28d = (((weaning BW - initial BW)/25 d); in period 29-56d = (((final BW - weaning BW)/28 d); in overall period = (((final BW - initial BW)/53 d)); ⁴ *FE* feed efficiency expressed as ADG (kg/d) to TDMI (kg/d) ratio; ⁵ *EPG* number of parasite oocysts/cysts per gram of feces; ^{a-b} Means within a column with different superscripts differ ($P \leq 0.05$).

Item	Treatment ¹				SEM	P-values		
	CON	MR	SF	MRS		Treatment	Period	Treatment x Period
Overall 3–56 d	1.16	1.08	1.14	1.09	0.01	0.28	< 0.001	0.034
EPG (x 10 ² /g) ⁵								
Initial (3 d)	0.47	0.45	0.42	0.44	0.01	0.39	-	-
Weaning (28 d)	2.12 ^a	1.46 ^b	1.74 ^{ab}	1.68 ^{ab}	0.03	0.05	-	-
Final (56 d)	0.86	0.55	0.76	0.65	0.01	0.25	-	-

¹ Treatment CON (control: without eubiotic feed additive in their milk replacer or their starter feed: n = 11); MR (eubiotic feed additive added to milk replacer: n = 11); SF (eubiotic feed additive added to starter feed: n = 11); MRS (eubiotic feed additive added to milk replacer and starter feed: n = 11); ² TDMI total dry matter intake from milk replacer and starter feed (kg/d); ³ ADG average daily gain (kg/d) in period 3-28d = (((weaning BW - initial BW)/25 d); in period 29-56d = (((final BW - weaning BW)/28 d); in overall period = (((final BW - initial BW)/53 d); ⁴ FE feed efficiency expressed as ADG (kg/d) to TDMI (kg/d) ratio; ⁵ EPG number of parasite oocysts/cysts per gram of feces; ^{a-b} Means within a column with different superscripts differ ($P \leq 0.05$).

Table 2

Different methods of eubiotic feed additive provision on occurrences of diarrhea by dairy calves

Item	Treatment ¹				SEM	P-values
	CON	MR	SF	MRS		
Diarrhea levels						
Score 1 (times)	42.7 ^b	49.9 ^a	45.2 ^{ab}	48.8 ^a	0.22	0.05
Score 2 (times)	2.80 ^a	1.80 ^b	2.50 ^{ab}	2.40 ^{ab}	0.07	0.05
Score 3 (times)	3.85 ^a	0.65 ^b	2.70 ^{ab}	0.85 ^b	0.04	0.05
Score 4 (times)	3.65 ^a	0.65 ^b	2.60 ^{ab}	0.85 ^b	0.01	0.05

¹ Treatment CON (control: without eubiotic feed additive in their milk replacer or their starter feed: n = 11); MR (eubiotic feed additive added to milk replacer: n = 11); SF (eubiotic feed additive added to starter feed: n = 11); MRS (eubiotic feed additive added to milk replacer and starter feed: n = 11); ^{a-b} Means within a column with different superscripts differ ($P \leq 0.05$).

Ruminal fluid pH, total VFA and N-NH₃ concentrations are presented in Table 3. The different methods of eubiotic feed additive provision had no effect on ruminal fluid pH ($P > 0.05$). The rumen fluid of the treatment groups differed in the total VFA and molar concentrations of propionate and butyrate ($P \leq$

0.05), and the MR treatment group had the highest levels of these indices. No relationships were detected between the different methods of eubiotic feed additive provision and concentrations of acetate, n-valerate, acetate to propionate (C₂:C₃) and butyrate to valerate (C₄:C₅) ratios and N-NH₃ (P > 0.05).

Table 3
Different methods of eubiotic feed additive provision on rumen fermentation in dairy calves

Item	Time (d) ²	Treatment ¹				SEM	P-values
		CON	MR	SF	MRS		
Ruminal pH	28	5.29	5.72	5.71	5.59	0.02	0.49
	56	6.04	6.20	6.44	6.21	0.08	0.53
VFA ³ molar concentrations (mmol/L)							
Total VFA	28	39.8	37.4	38.2	36.6	0.20	0.19
	56	70.6 ^b	79.2 ^a	75.3 ^{ab}	76.5 ^{ab}	0.11	0.05
Acetate	28	21.9	19.2	21.4	19.4	0.11	0.25
	56	32.2	32.9	35.7	34.5	0.09	0.25
Propionate	28	12.8	12.4	11.2	11.7	0.24	0.30
	56	27.7 ^b	32.2 ^a	27.3 ^b	29.3 ^{ab}	0.66	0.05
N-butyrate	28	3.76	4.70	4.35	4.26	0.06	0.19
	56	7.95 ^b	10.9 ^a	9.28 ^{ab}	9.48 ^{ab}	0.04	0.05
N-valerate	28	1.38	1.07	1.26	1.20	0.01	0.34
	56	2.75	3.18	3.06	3.22	0.04	0.25
C ₂ : C ₃ ratio ⁴	28	1.71	1.64	1.73	1.73	0.08	0.08
	56	1.16	1.12	1.11	1.26	0.02	0.25
C ₄ : C ₅ ratio ⁵	28	2.72	4.39	3.45	3.55	0.06	0.09
	56	2.89	3.42	3.03	2.94	0.06	0.42
NH ₃ -N (mmol/L)	28	21.2	13.2	19.5	17.5	1.38	0.16
	56	14.3	12.4	16.2	15.1	1.60	0.12

¹ Treatment CON (control: without eubiotic feed additive in their milk replacer or their starter feed: n = 11); MR (eubiotic feed additive added to milk replacer: n = 11); SF (eubiotic feed additive added to starter feed: n = 11); MRS (eubiotic feed additive added to milk replacer and starter feed: n = 11); ² Time age of calf (d); ³ VFA volatile fatty acids; ⁴ C₂ : C₃ ratio the ratio of ruminal acetate to propionate; ⁵ C₄ : C₅ ratio the ratio of ruminal butyrate to valerate; ^{a-b} Means within a column with different superscripts differ (P ≤ 0.05).

The blood metabolites concentrations are depicted in Table 4. All the results of the biochemical blood analyses were affected by the age of the calves, but statistically significant differences were detected

between treatment groups for concentrations of IGF-I and BHBA (during days 29–56 and over the entire experimental period; $P \leq 0.05$), and calves that were fed milk replacer containing eubiotic feed additive (MR treatment) had the higher values of these parameters.

Table 4

Different methods of eubiotic feed additive provision on biochemical blood indices in dairy calves

Item	Treatment ¹				SEM	P-values		
	CON	MR	SF	MRS		Treatment	Period	Treatment x Period
Insulin-like growth factor-I (ng/mL)								
Period 3–28 d	43.9	44.1	38.8	42.4	0.02	0.25	0.01	0.45
Period 29–56 d	37.7 ^b	53.4 ^a	39.2 ^b	39.3 ^b	0.01	0.05	0.01	0.56
Overall 3–56 d	41.4 ^b	51.0 ^a	38.4 ^b	41.2 ^b	0.03	0.05	0.01	0.59
β-hydroxybutyrate (mmol/L)								
Period 3–28 d	0.40	0.39	0.39	0.40	0.01	0.25	0.01	0.22
Period 29–56 d	0.51 ^b	0.62 ^a	0.50 ^b	0.53 ^b	0.01	0.05	0.01	0.23
Overall 3–56 d	0.45 ^b	0.53 ^a	0.45 ^b	0.45 ^b	0.01	0.05	0.01	0.25
Non-esterified fatty acids (mmol/L)								
Period 3–28 d	0.28	0.35	0.36	0.31	0.01	0.59	0.01	0.82
Period 29–56 d	0.30	0.31	0.38	0.30	0.01	0.60	0.01	0.62
Overall 3–56 d	0.29	0.33	0.37	0.31	0.01	0.59	0.01	0.51
Blood urea nitrogen (mg/dL)								
Period 3–28 d	8.43	8.43	8.30	8.20	0.02	0.63	0.01	0.28
Period 29–56 d	9.99	11.3	11.2	11.2	0.03	0.63	0.01	0.32
Overall 3–56 d	9.05	9.56	9.46	9.56	0.08	0.49	0.01	0.42
¹ Treatment CON (control: without eubiotic feed additive in their milk replacer or their starter feed: n = 11); MR (eubiotic feed additive added to milk replacer: n = 11); SF (eubiotic feed additive added to starter feed: n = 11); MRS (eubiotic feed additive added to milk replacer and starter feed: n = 11); ^{a-b} Means within a column with different superscripts differ ($P \leq 0.05$).								

Discussion

Considerable evidence of the potential effect of different kinds of natural feed additives on the health, growth performance, rumen fermentation, and biochemical blood indices of preweaned dairy calves is available in the literature; however, currently, only limited published scientific results are available on the complete comparison of the effects of all provision methods of feed additive on calf health, growth performance, fermentation and blood metabolites. In the current study, calves fed milk replacer containing eubiotic feed additive had better fecal score, EPG, and fewer occurrence of diarrhea (scores 3 and 4), which confirmed the better general health status of these dairy calves during preweaning period. Also, differences between groups were seen of the starter intake, and TDMI and calves that were provided with the eubiotic feed additive in their milk replacer (MR treatment) showed the greater effects. During the preweaning period calves of the MR treatment group consumed on average greater amounts of starter intake (0.54 vs. 0.37 kg/d) and TDMI (1.28 vs. 1.11 kg/d) in comparison to the CON group. Also, the calves in the MR group, weighed on average 7.0 kg more at the end of the study compared to the CON treatment (without eubiotic feed additive) and they gained 0.14 kg/d more weight during the preweaning period. These results show that feeding calves a milk replacer containing eubiotics with probiotics *Lactobacillus* spp. and the main bioactive compounds consisting of rosmarinic acid will enhance the growth performance, feed intake and calf health. The growth performance of young calves is strongly related to the type of feed which they consume, the rearing system and the intestinal microbiota balance. Probiotic and essential oils may prevent intestinal microbial imbalances, which are common in an intensive rearing system to reduce the incidence of disease. If calves become ill during the first few weeks of life, growth may decrease and results in death or poor productivity, even after they become adults [2, 22]. It is known that gut bacteria such as *Lactobacillus* spp. and *Bifidobacterium* spp. as well as *Faecalibacterium* spp. can modulate the immune system and inflammatory response, which can lead to metabolism alterations influencing feed intake, nutrient utilization, and growth performance [23]. Also, phytobiotics such as herbal extract of *Thymus vulgaris*, and *Oregano vulgaris* contain essential oils such as phenols (thymol, carvacrol, rosmarinic acid). These bioactive compounds have broad antimicrobial activity, particularly against gram-positive bacteria, by disrupting the bacterial cells membrane [24], which can lead to improved nutrient digestion [25]. Different mechanisms of action of probiotics and essential oils including rosmarinic acid have been described [12, 15, 17], which could be summarized as probiotics compete for nutrients and produce antibacterial compounds (e.g. VFA, hydrogen peroxide, nitric oxide, and bacteriocins) in the intestinal lumen allowing them to occupy specific niches of the intestinal mucosa and activate the innate immune system of calves [26]. Also, rosmarinic acid has antioxidant, antimicrobial (including bacteria, protozoa, and fungi), anti-inflammatory activity, and can act as an endocrine and immune stimulant [26]. The improvement of each of these mechanisms is directly related to improved calf health, feed intake, and nutrient utilization and thereby result in improved BW and ADG. In some studies, higher ADG was observed in calves that received probiotic and essential oils, mainly in the first two-three weeks of age [19, 28]. It is possible that, in the current study, the provided eubiotic feed additive within the milk replacer affected the calves so that they can faster accommodate to the stress of the first weeks of life, where they are not able to produce immunoglobulins in response to environmental stimulants for the first few days of their life; immunoglobulins appear at around 10–14 days of age [29]. In addition the natural feed additives within the eubiotics supports the immune system during this critical

period, which positively influences health, productive performance and metabolic status of the dairy calves during the preweaning period. Better starter intake and TDMI in calves fed eubiotics, incorporated into the milk replacer, may have resulted in an improvement of health and metabolic status, absorption of nutrients from the intestines, and faster rumen function development, which also improved growth performance such as BW and ADG. Also, the methods of feed additive provision may have influenced its effectiveness. The calves are mainly fed a milk replacer or whole milk and this is supplemented with calf starter feed during the preweaning period. Most published studies evaluated the provision of feed additive in the starter feed for dairy calves, to investigate benefits to rumen development and accelerate growth performance [19, 30, 31]. However, as seen in the current study, the intake of solid feed (calf starter) by the calves in the first 4 weeks of age is small [32, 33, 34], and the timing of the occurrence of enteric diseases is mainly in the first 30 days of life [35, 36]. Due to the calf's limited capability of ingesting large solid feed amounts in the first days of life, the supplement intake within the starter is limited during the early stages of the pre-weaning period and the desired supplementation level may not be achieved until later, which might possibly mask any effects. Similarly to our results (SF treatment), Seifzadeh et al., [37] showed that calves fed eubiotics consisting of herbal additives and probiotics mixed into the calf starter resulted in a lower intake of these additive in the first month because of the lower starter intake in comparison to the second month and therefore the effects of the herbal additives on growth performance was only observed in the second rearing month. On the other hand, in the current study, providing eubiotic feed additive to milk replacer and starter feed (MRS treatment) no influence on starter feed intake, growth performance, ruminal fermentation characteristics, and metabolic status. Thus far, no data have been published about the relationship between providing natural feed additives to both dietary offering methods (milk replacer and starter feed) on the above analyzed indices. The results are surprising and our hypothesis is that lower starter feed intake during the first month of calf life might possibly mask the effect of the effectiveness of eubiotic feed additive in the whole rearing period. Therefore, to increase effectiveness, especially in the first 4 weeks of life, the feed additives should be provided in the liquid feed [21]. Also, a meta-analysis conducted by Frizzo et al. [38] revealed that beneficial effects on calves' growth rate were observed when natural feed additives were added to milk replacer rather than to whole milk in the first few weeks of life, which was resulted in fewer health and nutritional problems. It is possible that the inclusion of the eubiotics during the first few weeks of the preweaning period should be mixed into the milk replacer and thereafter into starter feed, however, further investigations into potential causative mechanisms are needed.

The bioactive compound of essential oils and probiotics has prompted scientists to examine the potential to manipulate rumen microbial fermentation to improve feed intake and growth performance [39]. The start of ruminal fermentation can be noted at a very young age, and VFA can be found in the rumen of calves as early as the second week of life [40]. This is confirmed by enzymatic activities of ruminal microbiota (such as fibrolysis, amylolysis, proteolysis and ureolysis), which have been observed in the rumen from 4 to 10 day of age [40]. In the current study, provision of eubiotics within the milk replacer affected ruminal fermentation by increased concentrations of total VFA, propionate, and butyrate at the end of the preweaning period (d 56). Also, on d 28 and 56 of life, ruminal concentration of total VFA was

< 50 mmol/l and > 70 mmol/L, respectively, which is consistent with previous reports [41, 42]. Currently, there is no explanation for the mechanism of the improved rumen function after eubiotics provision *by-pass* on ruminal fermentation. It cannot be excluded that eubiotics, consisting of rosmarinic acid as the main bioactive compounds and *Lactobacillus* spp. probiotics, acted by other mechanisms, like altering the metabolism of the lower digestive tract, which could indirectly affect the rumen development as suggested for the mode of action in other feed additives such as sodium butyrate [43] or *Yarrowia lipolytica* yeast culture [33]. Also according to Hassan et al., [44] the functioning of reticular groove is less efficient as the calf ages, and in older calves part of the consumed milk can possibly enter the rumen and influence its fermentation. Rosmarinic acid, which is the main bioactive compound within the eubiotic feed additive, could cause hydrophobicity and disrupts bacterial membranes, increasing watery permeability and causing a toxic effect for the microorganisms [45]. This activity could result in inhibition of ruminal deamination and methanogenesis, which might affect the decrease in ruminal nitrogen ammonia, methane, acetate concentrations, acetate to propionate ratio, and an increase of the propionate and butyrate concentrations [46], which are important for ruminal papillae development, and especially propionate is used in the gluconeogenesis route. On the other hand, it could have been a consequence of the positive effect of the treatment on health of the animals, which may have stimulated, especially in older calves, to increase solid feed intake like starter feed and increased ruminal fermentation. Similarly to our results, Quigley et al. [47] showed a greater VFA concentration with greater feed intake and TDMI. Fermentation of calf starter feed increases ruminal concentration of VFA, especially propionate and butyrate, which most likely stimulates papillae development in the rumen [41]. Also, the major metabolic pathway of VFA metabolism in the rumen epithelium is ketogenesis [48]. The blood BHBA is produced by the metabolism of butyrate during its passage across the ruminal wall and, in consequence, higher levels of it can be used as an indicator of greater metabolic activity of ruminal epithelial cells [49]. Similarly, in the current study, ruminal butyrate and blood BHBA were higher during experiment respectively at d 28 and 56 and from d 29 to 56 in calves fed milk replacer containing eubiotic feed additive. Moreover, in MR treatment a greater blood IGF-I concentration was noted. IGF-I is a hormone produced in many tissues throughout the body, mostly in the liver [50]. It is a growth promoter that regulates the proliferation of many cell types, including epithelial cells of the intestine and rumen [51]. IGF-I is thought to be associated with the energy status of the body. In previous research, higher concentrations of this hormone in the serum corresponded with greater nutrient intake, enhanced growth and body weight [51]. It could have been a consequence of the positive effect of the MR treatment on animal growth and metabolic status, as rumen epithelial cells require an adequate supply of nutrients for their proliferation and differentiation.

Conclusion

Feeding calves a eubiotic feed additive provided into the milk replacer reduced the gut health challenges (diarrhea) and improved feed intake, growth performance, and enhanced ruminal fermentation of neonatal dairy calves. Feeding eubiotic feed additives in the liquid feed may provide a natural viable alternative to antibiotics to minimize health challenges, while improving calf growth performance. Also, to increase effectiveness, especially in the first 4 weeks of life, the feed additives should be provided

within the liquid feed, however, the biological significance of these results need to be investigated further in larger field trials.

Methods

Eubiotic feed additive characteristic

The experimental eubiotic feed additive consisted of combination of probiotic multi-strains of *Lactobacillus* spp. at a dose of 250 mg/calf/d and a phytobiotic, where the main bioactive compound was rosmarinic acid, at a dose of 50 mg/calf/d. The probiotics consisted of equal rations of three *Lactobacillus* species: *L. casei*, *L. salivarius* and *L. sakei* with a total of 10^{11} CFU/g. These strains were isolated from a healthy Holstein-Friesian calf in Poland and were manufactured by Poznan University of Life Sciences, Poland. These strains are patented with the following Genebank accession numbers: PKM B/00103, PKM B/00102, PKM B/00101. Further details about these strains has been published previously by Stefanska et al., [20]. The phytobiotic additive was prepared by the Institute of Natural Fibers and Medicinal Plants at the National Research Institute, Poznań, Poland and consisted of a watery extract of dried *Thymus vulgaris* and *Oregano vulgaris* to yield the experimental dose of rosmarinic acid, as the bioactive compound, at the level 50 mg/calf/d. The preparation details and experimental dose determination of the eubiotic feed additive were described by Stefanska et al., [20]. The stability of the eubiotic feed additive was assessed weekly, during storage.

Animals, Treatments And Management

This study used 44 Polish Holstein-Friesian calves. They were selected depending on sex (22 male and 22 female calves) and parity (22 each born from multiparous and primiparous cows) and were separated randomly into the four treatments groups consisting of 11 calves each for the duration of the study (56 days). The treatment groups differed by the method of the eubiotic feed additive was provided, the groups were: CON (control, without eubiotic feed additive in their milk replacer or their starter feed), MR (eubiotic feed additive added to milk replacer), SF (eubiotic feed additive added to starter feed), MRS (eubiotic feed additive added to milk replacer and starter feed). The eubiotic feed additive, supplied as dry powder, was mixed into milk replacer immediately before feeding and for the starter feed the eubiotic feed additive were mixed into the commercial mineral and vitamin premiks and then used to produce a pelleted starter feed provided during the experimental period. The calves, all obtained from a single commercial herd; they were separated from their mothers 2 h after birth and were placed into (2.9 m × 1.1 m × 1.8 m; length × width × height) individual pens containing wood sawdust bedding for the duration of the trial. Every day, the pens were refreshed by removing manure and adding new sawdust to make sure that the calves were in dry and clean environments. Physical contact between animals was minimized by using individual pens. Within 24 h after birth the calves received 4 L of high-quality (at least 50 g/L IgG concentration) colostrum [52], this was given in two feedings (< 2 h and < 12 h after birth). Between 24 and 48 h after birth, blood samples were taken from the jugular vein to determine the transfer of passive

immunity through measurement of initial serum total protein concentration (no. T7528, Pointe Scientific, Warsaw, Poland). The serum of all calves contained total protein concentrations of > 6.0 g/dL ($P > 0.05$). This indicated an adequate passive transfer of immunity [48]. On the 2nd and 3rd day the calves were given transition milk (4 L/d in 2 equal feedings at 9:00 AM and 5:00 PM). From day 4 until day 49, the calves were given 6 L/d of reconstituted milk replacer in equal amounts three times daily at 6.00 AM, 2.00 PM, and 8.00 PM. From day 50 until day 56 only 2 L milk replacer were offered once daily at 6.00 AM. The 150 g milk replacer powder (25 % CP, DM basic, and 18 % ether extract, DM basis, Polmass Milk, Bydgoszcz, Poland) were reconstituted with 1 L of water. Throughout the experiment, animals had constant access to fresh water, and water was changed daily. From day 4 onwards, calves were offered pelleted starter feed containing whole corn grain (77/23 w/w, 23 % CP, DM basic, Cargill, Kiszkowo, Poland) formulated according to National Research Council guidelines [53] every morning at 10:00 AM *ad libitum* with an excess of at least 10% (i.e. the amount of the starter, which was not consumed during the last 24 h). The excess starter feed was collected and weighed daily for each calf. The nutritional composition of the starter feed were analysed on a weekly basis for 8 representative samples were collected after morning feed as described by Stefanska et al., [20]. Procedures of the Association of Official Analytical Chemists [54] were used to analyze the samples for dry matter (DM, method no. 934.01), ether extract (EE, method no. 973.18), crude protein (CP, method no. 976.05), acid detergent fiber (ADF, method no. 973.18). The NDF was determined by the method described by van Soest et al. [55] and the concentrations of macroelements were measured by inductively emission (ICP-OES) in an Optima 2000 DV Spectrophotometer. The starch content of the starter feeds was determined according to the procedure of Hall [56]. The nutritional and chemical data for the milk replacer and starter feed are shown in Table 5.

Table 5
The nutritional value of milk replacer: and starter feed (mean \pm SD) on a DM basis

Nutritional value (%) ¹	Diet	
	Milk replacer	Starter feed
² CP	25.0	23.0 \pm 0.16
³ NDF	-	17.8 \pm 0.18
⁴ ADF	-	8.10 \pm 0.14
Starch	-	43.7 \pm 0.35
Ether extract	18.0	2.90 \pm 0.12
Ash	6.80	7.00 \pm 0.22
Calcium	0.84	0.80 \pm 0.08
Phosphorus	0.63	0.58 \pm 0.02
¹ The nutritional value of the milk replacer is according to the manufacturer's information. The representative samples of starter feed was collected weekly: immediately after the morning delivery: to determine their nutritional value (AOAC: 2010); ² CP crude protein; ³ NDF neutral detergent fiber; ⁴ ADF acid detergent fiber		

Feed intake and growth performance

During the study, calves were weighed on d 3 and then at weekly intervals from wk 1 to 8. Individual intake of starter feed was measured daily. For 3 experimental intervals (days 3 to 28, days 29 to 56 and days 3 to 56), average daily gain (ADG; calculated as final BW minus the initial BW divided by the number of days), the total dry matter intake (TDMI; from both the milk replacer and the starter feed), and feed efficiency (FE; AGD divided by TDMI) were determined. Individual calf biometric measurements were noted on a weekly basis, starting on d 3. This included body length (BL), heart girth (HG), hip width (HW), and height (HH) as described by Khan et al., [49]. A veterinarian, who was unaware of the animal groupings, throughout the experimental period, monitored the health of the calves daily. According to the standard operating procedure of the farm, dams were administered a total of 3 vaccinations of rotavirus and coronavirus at approximately d 30 and d 60 before calving and at 2 wk after calving. The consistency of feces was recorded every morning, before feeding milk replacer, using the following scoring system was used to measure fecal consistency: 1 = firm; 2 = soft or of moderate consistency; 3 = runny or mild diarrhea; and 4 = watery and profuse diarrhea [30]. Statistical analyses were conducted for individual calves using weekly averages for the fecal scores. The fecal score was used for the analysis of diarrhea incidence according to recommendations by Liu et al. [19]. Fecal scores \geq 3 were used for used to determine the incidence of diarrhea. Calves with diarrhea that lasted for \geq 24 h were treated twice daily orally using a stomach tube with manual vacuum pump until their fecal score was 2 or less. They received 1 L hydrating dextrose saline solution (glucose 6.23 g/L, sodium chloride 10.7 g/L, sodium

carbonate 2.69 g/L, potassium chloride 1.94 g/L) after which the milk replacer diet started again. During the study no calf died and no antibiotics were given.

Copro-parasitological analyses was performed, to determine the effect of parasites on the health of the calves. Fecal samples that were collected from the rectum of calves on d 3, 28 and 56. The microscopic analyses of the feces were conducted as described by Stefanska et al., [20].

Ruminal fluid sampling and analysis

On d 28 and 56 at about 2:00 PM (\pm 30 min), which is about 4 h post starter feeding, the rumen content (approximately 150 mL) of each calf was collected using a stomach tube with a manual vacuum pump. The process of collection and processing are described by Stefanska et al. [20].

Blood sample collection and analysis

On the first day of the study and then every 14 d throughout the study blood samples were collected from each calf from the jugular vein at 2:00 PM, which is about 4h (\pm 30 min) after feeding of the starter feed in the morning. The blood was collected into tubes containing polystyrene granules covered with a clotting activator (KABE, Poznan, Poland). The blood tubes were then transported to the laboratory, where they were processed and analyzed as described by Stefanska et al. [20]. The inter-and intra-assay variation was controlled by limiting the coefficient of variation to \leq 5% for all blood variables.

Statistical analyses

The MIXED procedure within the SAS software version 9.4 [57] was used to analysed the data. The UNIVARIATE procedure of SAS was used test the normality of the data before any further analyses were carried out. Using a logistic transformation function the fecal score, the total number of parasite oocysts/cysts per gram of feces, and diarrhea occurrence were transformed before statistical analysis. The MIXED procedure was used to analyze the starter intake, growth performance, fecal score, and blood metabolites data for three periods: d 3 to 28; d 29 to 56, and the overall experimental period from d 3 to 56, using the following model: $Y_{ijklm} = \mu + l_i + m_j + p_k + t_l (p \times t)_{kl} + e_{ijklm}$ where: Y_{ijklm} – is the dependent variable, μ – is the average experimental value, l_i – is the random effect of parity of dam (i = is primiparous cows or multiparous cows), m_j – is random effect of sex of calf (j = is male or female), p_k – is the fixed effect of measurement period (k = is the number of 14-days measurement periods), t_l – is the fixed effect of treatment (l = CON, MR, SF or MRS), $(p \times t)_{kl}$ – is the interaction of period \times treatment, and e_{ijklm} – is the error term. In the MIXED MODEL, the fixed effects were period, treatment, and treatment by period interaction and the random effects were dam parity and calf sex. The covariance structures that were tested included CS, Simple, UN, TOEP, AR (1), ARH (1), and ANTE (1) to find the best-fitted structure for the model. A 14-day measurement period of was modeled as a repeated measurement by using the compound symmetry as the covariance structure on the basis of best fit determined by the lowest Bayesian information criterion. In cases of significant treatment, individual comparisons were made

using Duncan's adjustment. Statistical significance was declared when $P \leq 0.05$ and trends were indicated when $0.05 < P \leq 0.1$.

Data on body weight, EPG, diarrhea occurrences, and rumen fermentation characteristics were subjected to ANOVA according to the following model: $Y_{ij} = \mu + \text{Treatment}_i + e_j$ where: Y_{ij} – is the dependent variable; μ – is the average experimental value; Treatment_i – is the effect of treatment ($i = \text{CON, MR, SF or MRS}$); e_j – is the error term.

Abbreviations

ADF

acid detergent fiber; ADG:average daily gain; BHBA: β -hydroxybutyrate; BL:body length; BUN:blood urea nitrogen; BW:body weight; CON:control group:without eubiotic feed additive in their milk replacer or their starter feed; CP:crude protein; DM:dry matter; EE:ether extract; EPG:number of parasite oocysts/cysts per gram of feces; FE:feed efficiency; HG:heart girth; HH:hip height; HW:hip width; IGF-I:insulin-like growth factor-I; MR:eubiotic feed additives added to the milk replacer; MRS:eubiotic feed additives added to the milk replacer and the starter feed; NDF:neutral detergent fibre; NEFA:non-esterified fatty acids; N-NH₃:ruminal ammonia nitrogen; SF:eubiotic feed additives added to the starter feed; TDMI:total dry matter intake; VFA:volatile fatty acids.

Declarations

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Authors contributions

BS methodology, formal analysis, interpretation of the data, investigation, writing original draft, writing - review and editing. FK data curation, interpretation of the data, language correction, writing - original draft, writing review and editing. BG writing - original draft, writing review and editing. SS writing - original draft, writing review and editing. PS software, writing - original draft, editing. AF funding acquisition. WN conceptualization, investigation, writing original draft, writing - review and editing. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

All animal procedures: conducted for this study are in accordance with the "Act on the protection of animals used for scientific purpose" of the Republic of Poland: which is fully compliant with the EU directive no. 2010/63/EU [58] for the protection of animals used for scientific purposes and were approved by the Local Ethical Committee for Experiments on Animals in Poznań, Poznań University of Life Sciences (No. 21/2015). The authors obtained written informed consent to use the animals in the experiment from the owner of the farm.

Consent for publication

Not applicable

Competing interests

The authors declare no competing of interest.

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