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Whey permeate-derived milk acidifier for dairy calves

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Abstract. A milk acidifier obtained from whey permeate fermenting it with dairy propionic acid bacteria was tested in this study to evaluate the effects of milk acidification on the health and growth performance of pre-weaned dairy calves. The study consisted of 30 neonatal Holstein female calves, allocated to three treatments fed unacidified (Control group) or acidified (EG-1 and EG-2 groups) pasteurised milk during the 7–75 day age. Control and EG-1 were fed milk by divided method three times daily till one month of age, then twice daily until weaning; EG-2 was basically fed by the undivided method - one week three times daily (7–14 day age), then once daily. Results demonstrate that animal general health status and faecal scores (FS) were good and the tested acidifier can be used for pre-weaned calf milk acidification. Biochemical and haematological indices of blood at the 30 and 60 day age were within normal reference values with both - divided and undivided - milk feeding methods. Mean live weight (LW; 106.6 ± 9.40 kg on average) and live weight gain (LWG; 911.33 ± 109.04 g day^{-1} on average) at weaning did not differ between treatments ($P > 0.05$). Lower intake of starter feed associated with a larger amount of milk consumed was observed in EG-2 animals ($P < 0.05$). As the results observed regarding growth performance and health indices of all dietary treatment groups of calves were similar, we could anticipate that the acidification benefits would be greater when providing unpasteurised milk, or during the hottest weather when the risks of milk spoilage are greater.

Key words: blood; growth performance; heifer calves; propionic acid bacteria.

INTRODUCTION

Digestive problems, e.g., diarrhoea, is the most frequently encountered health problem in young dairy calves prior to weaning (Breen et al., 2012; House et al., 2015, Zhang et al., 2019). In recent years there is growing interest in promoting animal health by natural means of disease prevention to reduce the need for antibiotics and to stabilise gut health (Cardo, 2016; Celi et al., 2017; EC, 2017; Dai et al., 2021). The microbiological quality of the milk fed to the young cattle is of great importance because milk is the main food for calves until weaning, which is usually done at 2–3 months of age. Milk acidification is a preventive method to diminish the use of antibiotics, whose overuse to treat or prevent diarrhoea has led to the development of antibiotic resistance endangering animal and human health. Acidification is a fast and technically simple method, often recommended when pasteurisation and cooling are not practicable; it saves the time and labour required for feeding as well (Coelho et al., 2020). Materials used to acidify animal feed vary - most often these are concentrated organic acids - formic (Li et al., 2019; Chen et al., 2020), hydrochloric (Zhang et al., 2017), lactic (Coelho et al., 2020) and other acids. Propionic acid can also be used for milk acidification purposes; however, when used alone, the high corrosivity and low palatability may limit its usefulness (Jones & Heinrichs, 2014). It is a 3 carbon organic acid, possessing valuable antimicrobial properties (Ranaei et al., 2020), obtained mainly by petrochemical routes (Gupta & Srivastava, 2001; Ali et al., 2021). The availability of fossil resources is declining, while for a food by-product - whey - it is often difficult to find a use. Whey is an abundant and bulky dairy by-product arising from cheese and curd manufacture. Due to high biochemical oxygen demand, the disposal of whey in water streams is not allowed as such poses a high environmental threat (Audic et al., 2003, Bargeman, 2003; El-Tanboly et al., 2017). Meanwhile, due to high sugar lactose content, whey is a good substrate for organic acid production (Audic et al., 2003). Sometimes, in relatively small quantities, whey is used in animal nutrition including ruminant and calf feed (Manurung, 2012; El-Shewy, 2016; El-Tanboly et al., 2017; EWPA, 2020). Therefore, we were looking for new uses for this by-product in animal feed. We fermented whey permeate with dairy propionic acid bacteria and obtained an acidifier for calf's milk. This microorganism species is often used in the production of cheese; some its strains have QPS (Qualified presumption of safety) status (European Food Safety Authority, 2012; Rabah et al., 2017). It is also added to silage as a technological additive, or used as probiotics (Adams et al., 2008; Rabah et al., 2017) and protective microorganisms in a variety of foods due to the antimicrobial properties of their metabolites (Gupta & Srivastava, 2001; Zarate et al., 2011; Poonam et al., 2012; Bai & Rai, 2015; Azzaz et al., 2019). Although the idea of animal feed acidification is not new (Diebold & Eidelsburger, 2006; Partanen & Mroz, 1999; Zou et al., 2017; Long et al., 2018; Coelho et al., 2020), the use of whey to produce acidifier for calves' milk would improve the recycling of dairy industry by-products and lessen the overall environmental burden. The fermented whey products can also serve as functional feed components - a source of B group vitamins, microbial protein, and energy (Widyastuti & Febrisiantosa, 2014; Poonam et al., 2012). Thus, the aim of the present study was to investigate the effects of the new whey-derived milk acidifier on the health and growth performance of dairy calves in a divided and undivided milk feeding system.

MATERIALS AND METHODS

Animals, Management and Feeding

The experiment was conducted at the dairy farm 'Līgotnes' of the Training and Research farm SIA 'Vecauce' of LLU (Dobele district, Latvia); it was approved by the Animal Welfare and Protection Ethics Council of the LLU (permit LLU DZLAP No. 20/3). Test groups were established gradually, subdividing 30 neonatal Holstein female calves, born during the August and September months, into three dietary treatment groups with 10 replicates in each - EG-1, EG-2 and Control. The inclusion of animals in the test groups was decided based on birth weight and health status. This study lasted until December of the same year. Schematic description of milk provided to the calves and its feeding methods are presented in Table 1.

Table 1. Schematic description of milk provided to the calves and its feeding methods

Age of calves (days)	Dietary treatment groups		
	EG-1 (n = 10)	EG-2 (n = 10)	Control (n = 10)
1–4	1. Colostrum and transition milk feeding, similar to all calves Warm (35–38 °C) colostrum or cow transition milk fed 3 times daily (6 ± 1.3 L day^{-1} on average) ↓		
5–6	2. Milk feeding, similar to all calves Pasteurised (72–76 °C, 15–20 s) warm (35–38 °C) bulk milk fed 3 times daily (8 ± 0.8 L day^{-1} on average) ↓		
	3. Milk feeding, diversified (8 ± 0.3 L day^{-1} from day 7 till 66; 6 ± 1.4 L day^{-1} from day 67 till 72; 3 ± 1.1 L day^{-1} from day 73 till 75 on average):		
	acidified pasteurised bulk milk; pH 4.6–5.2; t = 20–25 °C	**acidified** pasteurised bulk milk pH 4.2–4.6; t = 20–25 °C	**un-acidified** pasteurised bulk milk pH 6.5; t = 35–38 °C
7–14	fed three times daily till 1 month of age (divided method)	fed three times daily till 14 days of age (divided method)	fed three times daily till 1 month of age (divided method)
15–30		fed once a day (undivided method)	
31–75	fed twice daily (divided method)		fed twice daily (divided method)

After birth, each newborn animal was placed in a special pen with a heating lamp for drying, and on the first time of feeding was fed on colostrum within two hours after birth. After drying, the calves were transferred to the outdoor shed and housed under equal conditions in welfare-sized, individual wooden pens bedded with straw. Before inserting the calves, the cages were cleaned and bleached; the base was disinfected and supplemented with sand and straw. Later, the cages were cleaned and topped up with straw as needed. Starting from 24 to 48 hours after birth for 7 consecutive days, the calves were given a prophylactic oral solution containing the active substance halofuginone (Kriptazen, Virbac, France, administered PO according to the manufacturer's instructions) to prevent *cryptosporidium*-induced diarrhoea. All calves on the first feeding, within 2 hours after birth, received colostrum (only once), the volume of which was 10–12% of

the body weight. Calves received colostrum from their respective dams if the quality was adequate (solids content ≥ 22% Brix as determined by hand refractometer). In the case when milking staff was not present, e.g., at night, and therefore no mother milk was available, or there was no mother milk available of adequate quality, thawed colostrum of confirmed quality (previously stored at −20 °C) was provided. Later, up to the 4th day of life (inclusive), calves were fed transition milk. On days 5 and 6 calves were fed pasteurised bulk milk from the same farm. Milk was pasteurised and cooled to the necessary temperature in a water bath. Colostrum and milk was provided to calves from a bucket with a nipple. Until the 6 day age, the temperature of milk fed to all calves was 35–38 °C. From the first day of life, drinking water was available in a separate bucket. From the 4th day, commercial calf starter meal (concentrates), containing vitamins A, D_3, E and microelements, including iron, copper, zinc, and hay was provided *ad libitum*.

From day 7, feeding was diversified by feeding acidified or unacidified whole bulk milk. Control group calves were fed unacidified warm (35–38 °C) milk 3 times daily till one month of age, then twice daily. Group EG-1 calves were fed cool acidified milk (20–25 °C) 3 times daily till one month of age, then twice daily by split or divided method. Group EG-2 calves were fed cool (20–25 °C), stronger acidified milk 3 times a day from the 7th to the 14th day, then once a day (by undivided method) from the 15 day age till weaning. When the undivided/unrestricted method for the EG-2 group animals was applied, the daily milk intake in the morning (4:00), i.e., 8 L day^{-1} per calf was offered. If the calf had consumed the required amount of milk by 16:00, during the 1st month of life it was offered an additional portion of milk (1–3 litres). At the end of the day, the amount of milk consumed by each calf on that day was recorded by measuring the quantity of unconsumed milk. Milk acidification was performed by adding acidifier to cool milk (20–25 °C) in doses sufficient to achieve the desired acidity of the final product and to coagulate it. The target pH of the milk fed in the restricted feeding group (EG-1) was higher (pH 4.6–5.2) because the feeding time of the milk was relatively short (20 min.) and the risk of microbial damage was lower, while the pH of the milk fed in the EG-2 group was lower (target pH 4.2–4.6) - stronger acidification was needed due to the longer feeding time of the milk (approximated method to unlimited milk feeding). For the calves to be gradually accustomed to acidified milk, during the first 5 days the dose of acidifier was gradually increased from 15 mL to 33 mL (EG-1) or to 41 mL (EG-2) per 1 L of milk, at the same time decreasing the temperature of the milk from 35–38 °C to 20–25 °C. Over the 7–66 day age, calves received 8 L milk day^{-1} on average. Starting from 67 up to 75 days of age, calves were gradually weaned, reducing the milk dose. The weaning was finished considering the following praxis: 1) when doubling of calves' live weight according to calf breeding experts was reached, and 2) when starter intake according to starter meal producers' instruction was at least 1 kg for 3 consecutive days.

Main feeding materials of calves are listed in the Table 2. The main raw material of the acidifier tested in the study was milk whey permeate (> 90%), obtained in a milk processing plant, and fermented under laboratory pilot scale conditions at the Faculty of Food Technology of the LLU, using appropriate equipment to ensure sterility and other work safety measures.

Cultures of propionic acid bacterium (DSM 20273, 20535, 16859, and 4902) were obtained from the Leibniz Institute DSMZ culture collection (German Collection of Microorganisms and Cell Cultures, GmbH). Bacterial maintenance and propagation of

pure cultures was carried as described by Antone et al. (2021). For product manufacture and stability, yeast extract, salt additives and feed grade formic acid was used. The product (pH 2.3) was ready for adding to milk and did not need to be diluted.

Table 2. Main feeding materials

Items	Fat, %	Protein, %	Lactose, %	DM, %	Crude fibre, %	Crude ash, %	NDF, %
Milk (Control group)	4.41	3.51	4.53	13.40	-	-	-
Milk (EG-1 group)	4.27	3.42	4.68	13.36	-	-	-
Milk (EG-2 group)	4.24	3.40	4.72	13.34	-	-	-
Starter meal for 0–1 month old calves*	4.00	19.40	-	-	5.30	7.50	-
Starter meal for 30–75 day old calves**	2.68	20.00	-	-	4.06	7.15	-
Hay	-	13.64	-	85.04	36.14	6.05	61.64
Milk acidifier	0.03	0.63	9.30	12.00	-	-	-

DM – dry matter; NDF – neutral detergent fiber ;*'Danish Calves Crunch' (Vilomix Baltic; mineral content: Ca 1.07%, P 0.41%, Na 0.31%, Mg 0.21%); ** 'Supplementary feed for calves with linseed' (Dobeles Dzirnavnieks; mineral content: Ca 1.00%, P 0.60%, Na 0.30%, Mg 0.29%).

General Health Status and Growth Performance Assessment of Animals

The general health status and clinical signs of disease of the calves were monitored daily by a veterinarian, checking the general appearance, animal activity, appetite, and appearance (colour and consistency) of faeces. Calf live weight (LW) was recorded after birth and later at 15 day intervals: on day 15, 30, 45, 60, 75, and 90. LW of experimental animals at birth was similar in all groups ($P > 0.05$) (Table 6). Calves were weighed on an electronic calibrated scale. All weights were taken approximately 3 h after the morning milk supply. Average live weight gain (LWG) was calculated by subtracting the initial LW from the LW at the end of the period and divided by the number of days. Faecal score (FS) data were recorded daily 2 h after the morning milk feeding. Faeces were scored on a 0 to 4 point scale according to the method used by Teixeira et al. (2015) based on visual consistency and colour evaluation, where: 0 = normally formed faeces with normal colour; 1 = pasty with normal colour; 2 = liquid with normal colour; 3 = watery with normal colour; 4 = watery with abnormal colour. According to the methodology, if the FS was more than or equal to 3, the animal was considered to have diarrhoea.

Feed Intake and Feed Quality Measurements

Milk and starter feed intake was recorded daily. Milk volume was measured with the help of a measuring cup. Milk was sampled daily and frozen at −20 °C for fat, crude protein, lactose, and total solids content analyses by a MilkoScan™ Mars analyser (Foss Electric, Denmark) and detection of pH (pH meter Jenway, Model 3510, Cole-Parmer, Staffordshire, UK). The starter was weighed on a scale.

Blood Biochemical and Haematological Parameters

For the determination of general blood biochemical parameters, blood samples were taken at 6, 30 and 60 days of age. Collection was carried out in the morning, two hours after feeding. Samples were taken from the jugular vein by venipuncture. For

analysis of blood serum biochemical indices (aspartate aminotransferase (AST), alkaline phosphatase (ALP), urea, creatinine, total serum protein (TP), albumin, and glucose) samples were taken in non-EDTA vacuum tubes, and transported under refrigeration (2–4 °C) to the Laboratory within three hours after collection. Biochemical analyses were made using automated methods with ABBOTT ARCHITECT analytical equipment (Abbott, Illinois, U.S.A.). For the haematological analyses (erythrocyte count (E), haemoglobin (Hb), and haematocrit (Hct)) samples were collected into EDTA vacuum tubes. Blood samples were tested with the veterinary haematological analyser Exigo EOS Vet (Boule Medical AB, Sweden) having built-in electrical resistance or impedance method for E, and cyanide-free spectrophotometrical method (at 535 ± 5 nm) for Hb. Hct was calculated arithmetically.

Statistical Analysis

The assumption of normal data distribution was assessed by Shapiro-Wilk's test and a visual inspection of their histograms and normal Q-Q plots. The assumption of homogeneity of variances was tested by Levene's test. To determine whether there were any statistically significant differences between the three independent groups, we used a one-way ANOVA with Tukey post hoc comparison or Kruskal–Wallis h test with pair-wise comparisons using Dunn's procedure (1964) with a Bonferroni adjustment. Statistical data analysis was carried out using SPSS Statistics version 23 (IBM Corporation, Chicago, Illinois). When the shape of data distribution was not similar between independent groups the mean ranks were calculated to present the obtained results (Vargha & Delaney, 1998). For the calculation of statistically significant differences of LW and LWG between test groups, the MS Excel program was used. To test the difference between two independent groups the independent samples T-test was used. Values of less than 0.05 ($P < 0.05$) were considered significant. Data are presented as *means* ± *SD* unless otherwise stated.

RESULTS AND DISCUSSION

Health Status and Growth Performance of Animals

The overall animal health status was good - the general appearance of calves was normal and alert; appetite was good and satisfactory. No serious health problems were observed during the study. Results of blood biochemical parameters and their reference values of some other studies are listed in Table 3. Most biochemical indicators - serum AST and ALP activity, also TP, albumin, creatinine, and glucose concentrations - at each individual sampling time - at the first sampling before the start of milk acidification, as well as later - at the age of 30 and 60 days, were similar ($P > 0.05$) in all dietary treatment groups. The only difference was significantly lower ($P < 0.05$) relative to the Control, still normal compared to literature data, urea concentration at 2 months of age in EG-2 serum. ***Serum AST*** activity in all dietary treatment groups was similar to reference values as observed by Ježek (2007) and Yu et al. (2019). Significant increase in AST in the blood of all groups of animals at 1 month and 2 months of age compared to the 6 day age was observed. Since this indices was within the normal reference range, it can be concluded that the animals were not significantly affected by muscle or liver damage (Stämpfli & Espinosa, 2015).

Table 3. Blood serum biochemical parameters of differently fed calves groups

Indices	Groups; *p*-values; Reference values	Median values and intervals (Q1–Q3) in the respective age of the animals (days)		
		6	30	60
AST, U L^{-1}	EG-1	**33.5** (31.0–37.3)	**33.5** (30.0–34.8)	**47.5** (40.3–53.8)
	EG-2	**31.5** (29.3–34.8)	**32.5** (31.3–36.5)	**41.5** (40.0–47.8)
	Control	**34.0** (31.5–37.0)	**33.5** (29.5–38.8)	**46.5** (41.3–49.0)
	p-values	0.725	0.966	0.278
	Reference values	38.7 ± 16.1*	33.3 ± 11.0**	40.3 ± 10.2***
		36.6 ± 9.3 ^	42.2 ± 15.0^^	37.5 ± 10.8^^^
ALP, U L^{-1}	EG-1	**377.5** (276.8–401.8)	**480.5** (362.8–572.0)	**615.5** (524.3–733.3)
	EG-2	**323.0** (276.8 –483.0)	**477.5** (399.0–601.3)	**512.0** (434.0–558.5)
	Control	**324.0** (281.8–405.0)	**421.5** (331.0–792.3)	**439.5** (350.0–560.3)
	p-values	0.961	0.889	0.064
	Reference values	262.2 ± 185*	157.8 ± 79.7**	216.2 ± 97.7***
		~780[§]	~260[§§]	~410, 580[§§§]
Total protein, g L^{-1}	EG-1	**61.5** (58.3–64.8)	**57.0** (56.0–59.0)	**60.5** (59.0–61.8)
	EG-2	**64.5** (60.8–65.8)	**57.5** (55.3–60.8)	**59.0** (57.3–60.5)
	Control	**63.0** (60.0–70.0)	**59.0** (58.0–62.3)	**61.0** (60.0–61.8)
	p-values	0.810	0.180	0.094
	Reference values	52.09 ± 7.09*	52.41 ± 3.91**	56.94 ± 5.19***
		51.1 ± 6.3^	49.9 ± 5.0^^	48.0 ± 5.4^^^
Albumin, g L^{-1}	EG-1	**22.0** (21.0–22.0)	**27.0** (26.3–28.0)	**29.0** (29.0–30.0)
	EG-2	**22.0** (20.0–23.0)	**27.5** (27.0–29.0)	**29.5** (29.0–30.0)
	Control	**22.0** (21.3–23.0)	**28.0** (26.3–28.0)	**29.0** (28.0–30.0)
	p-values	0.668	0.647	0.840
	Reference values	26.35 ± 2.10*	30.99 ± 2.53**	31.76 ± 2.68***
		~28[§]	~35[§§]	~37, 36[§§§]
Urea, mmol L^{-1}	EG-1	**3.65** (3.23–4.10)	**3.80** (3.50–4.35)	**2.60**[ab] (2.42–2.85)
	EG-2	**3.25** (2.70–4.50)	**3.35** (2.92–3.75)	**2.35**[b] (2.02–2.60)
	Control	**3.30** (3.13–3.90)	**3.05** (2.55–3.58)	**2.80**[a] (2.50–3.18)
	p-values	0.845	0.083	0.036
	Reference values	3.64 ± 1.33*	4.01 ± 1.21**	3.90 ± 1.19***
		2.53 ± 1.32^	2.31 ± 0.65^^	1.86 ± 0.48^^^
Creati-nine, µmol L^{-1}	EG-1	**71.5** (65.3–77.0)	**63.0** (60.5–64.0)	**58.5** (57.0–62.0)
	EG-2	**64.5** (62.0–69.8)	**61.0** (58.75–62.5)	**59.5** (56.0–64.5)
	Control	**70.5** (66.5–86.8)	**68.5** (64.8–71.8)	**58.0** (53.0–62.5)
	p-values	0.178	0.089	0.790
	Reference values	113.76 ± 36.20*	93.55 ± 16.66**	82.34 ± 15.10***
		88.42 ± 17.68^	73.39 ± 14.14^^	59.24 ± 10.61^^^
Glucose, mmol L^{-1}	EG-1	**6.61** (5.95–7.66)	**6.21** (6.04–6.76)	**6.25** (6.18–6.64)
	EG-2	**6.85** (6.12–7.37)	**6.28** (5.97–6.94)	**6.25** (6.03–6.70)
	Control	**6.35** (6.15–6.52)	**6.96** (6.33–7.03)	**6.07** (5.55–6.31)
	p-values	0.218	0.624	0.365
	Reference values	6.14 ± 1.23^	5.66 ± 1.02^^	5.17 ± 0.85^^^
		6.2 ± 1.3 (treatment group) and 6.2 ± 1.7 (control)[#]		

[a, b] values with different superscript letters on the same column at each blood indicator are different according to the Kruskal-Wallis h *test* with pair wise comparisons using Dunn's procedure ($P < 0.05$); 1*, 4**, and 8*** week old Holstein-Friesian calves (Ježek, 2007); 2^, 5^^ and 7^^^ week old male Holstein calves, *mean* ± *SD* (Yu et al., 2019); 24–48h[§], 28d[§§], 56 and 70d[§§§] old Holstein calves (Mohri et al., 2007); [#] 0, 4, 8, and 12-week old Holstein-Friesian bull calves; *mean* ± *SD* (Adams et al., 2008).

Serum ALP activity at the 6 day age in calves' blood of the present study was within normal reference values as observed by Ježek (2007), but lower than Mohri et al. (2007). Later, there was a statistically significant ($P < 0.05$) increase in ALP activity in the blood of all groups of animals at 2 months of age compared to the 6 day and 1 month age. At the age of 1 month the ALP activity in all test groups, but at the 2 month age, especially in experimental groups animals, was higher than in the above-mentioned research. At the age of 1 and 2 months the ALP activity of our experimental groups was similar to Knowles et al. (2000), whose calves' blood serum ALP concentration was around 500–620 U L^{-1}. The ALP activity in young animals can be up to 3 times higher than in adult animals because of the osteoblastic activity during rapid bone growth Bain (2011); up to the age of 6 months it can reach 1,800 U L^{-1} (Kraft, 1999). ALP enzyme comes from the placenta, bone, macrophages, intestinal epithelium, and liver. In very young calves, ALP is increased, probably because of the placental or bone source. In young calves, ALP levels up to 1,000 IU L^{-1} at birth and 500 IU L^{-1} at several weeks of age should be considered normal (Aiello & Moses, 2016). ***TP concentration*** in calves' blood at each individual sampling time in all the 3 dietary treatment groups was similar ($P > 0.05$). Compared to literature data, at the 6 day and 1 month age it was higher than the data of Ježek (2007) of 1 and 4 week old calves (52.09 ± 7.09 and 52.41 ± 3.91 g L^{-1}, respectively), but similar to Knowles et al. (2000) values (~62–68 and ~56–59 g L^{-1}). TP concentration in calves' blood serum may be within 50–70 g L^{-1} (Kraft & Dürr, 1999). At the 2 month age TP was within normal reference values as observed by Knowles et al. (2000) - ~58–61 g L^{-1} and Ježek (2007) - 56.94 ± 5.19 g L^{-1}. ***Serum albumin*** concentration at 6 days of age was relatively low - near the bottom line of the 22–36 g L^{-1} reference range of Kessell (2015), and lower compared to Ježek (2007), and Mohri et al. (2007) (Table 3). Over time, the levels of albumin increased ($P < 0.05$), similarly to that observed in the research of Knowles et al. (2000), Mohri et al. (2007) and Ježek (2007). Attention should be paid to the fact that blood levels of albumin in neonatal calves of the present study were relatively low. Decreased concentration of albumin could be associated with different causes, e.g., mild malnutrition, reduced absorption of amino acids in gastrointestinal tract, reduced synthesis of albumin in the liver or due to the invasion of *Cryptosporidium parvum* (Eckersall & Proteins, 2008). ***Serum urea*** concentration in all group calves' blood of the present study was similar to the concentrations assessed by Yu et al. (2019) and Ježek (2007) (Table 3). Urea and creatinine are waste products of protein catabolism (Kessel, 2015). ***Creatinine*** concentration in calves' blood serum of the present study was within normal reference values, similar as assessed in the study of Yu et al. (2019), but lower compared to Ježek (2007). ***Serum glucose*** concentration of all dietary treatment groups was within normal reference values as observed by Ježek (2007) and Adams et al. (2008). This shows the good energy intake status of animals throughout the study (Stämpfli & Espinosa, 2015). ***Haematological parameter (E, Hb, and Hct)*** results and their reference values are listed in Table 4. At both blood samplings no differences were found between groups ($P > 0.05$) and were within normal reference values as assessed in the study of Ježek (2007), except the Hct level at 2 month age. Yet it was similar to Li et al. (2019) study values (23–26%) of 70 day old female Holstein calves fed reconstituted, acidified reconstituted or acidified fresh milk. Regarding changes with age during the experiment there was a significant increase in E in the blood of all groups of animals ($P < 0.05$) that is also consistent with previous studies of Ježek (2007), and Mohri et al. (2007).

Table 4. Blood serum haematological parameters of differently fed calf groups

Indices	Groups; *p*-Values; Reference values	Median values and intervals (Q1–Q3) in the respective age of the animals (days)	
		6	60
Erythrocytes, $10e^6$ μL^{-1}	EG-1	6.330 (6.20–6.76)	8.880 (8.69–9.59)
	EG-2	6.320 (5.45–7.04)	8.830 (8.31–9.28)
	Control	6.620 (5.95–7.63)	9.090 (8.46–9.44)
	p-Values	0.663	0.842
	Reference values	7.63 ± 1.49*	8.53 ± 1.01**
Haemoglobin, g dL^{-1}	EG-1	8.90 (8.35–9.40)	9.25 (8.88–9.88)
	EG-2	9.00 (7.78–10.10)	9.25 (8.45–9.50)
	Control	9.85 (8.72–11.12)	9.35 (9.30–10.07)
	p-Values	0.220	0.620
	Reference values	10.41 ± 2.21*	9.83 ± 1.29**
Haematocrit, %	EG-1	27.60 (26.85–29.63)	25.10 (24.00–26.35)
	EG-2	26.85 (22.90–29.53)	24.45 (22.68–25.28)
	Control	27.80 (25.00–35.02)	25.20 (24.55–26.82)
	p-Values	0.673	0.561
	Reference values	30 ± 7*	30 ± 5**

1* and 8** week old Holstein-Friesian calves (Ježek, 2007).

In summary, normal compared to reference values, indices of blood show that the health status of animals throughout the study was good. No differences regarding biochemical and haematological indicators at each individual sampling time were observed between groups, except for significantly lower urea concentration at 2 months of age in the EG-2 serum ($P < 0.05$) compared to the Control, still normal compared to literature data (Ježek 2007; Yu et al. 2019). Hence, the animal blood parameters were not largely affected by the feeding of acidified milk or its feeding method.

Faecal score (FS) test showed no cases of diarrhoea - no animals had watery faecal output with FS above 2 points. As most commonly the faecal mass of animals was normally formed and coloured, consequently, calculated median values of all test groups resulted in the score of 0 for all time periods; to show the differences more clearly, results are presented in Table 5 as mean ranks. During the initial 1–15 day period after birth the mean rank of the EG-2 score was slightly but significantly higher ($P < 0.05$) - the consistency of the faecal masses was more liquid compared to the Control, but did not differ from the EG-1 ($P > 0.05$). In the course of the next 16–30 and 31–45 day periods FS in all groups were similar ($P > 0.05$). Later - around the2 month age - FS significantly differed ($P < 0.05$) between both experimental groups, over the 46–60 day period being slightly higher in EG-1 and over the 61–75 day period in EG-2, yet at the same time being similar ($P > 0.05$) to the Control. In some animals there were short-term

Table 5. Faecal score test results of dairy calves fed different types of milk

Age, days	Dietary treatments			*p*-value
	EG-1	EG-2	Control	
1–15	222.25[ab]	240.17[b]	214.07[a]	0.030
16–30	223.99	223.98	223.89	0.093
31–45	224.78	225.01	224.99	0.072
46–60	229.99[a]	222.50[b]	224.01[ab]	0.030
61–75	222.50[a]	229.98[b]	224.02[ab]	0.030

[a, b] values with different superscript letters on the same line are different according to the Kruskal-Wallis h test with pair wise comparisons using Dunn's procedure ($P < 0.05$); data are presented as mean ranks.

(up to 2 days) faecal consistency changes to liquid (FS 1–2 points); thus, the possibility of subclinical inflammation of the gut cannot be ruled out.

A looser faecal consistency can also often be associated with increased starter intake causing minor intestinal dysfunction because the microflora of the intestinal tract has not yet matured, and habituation to solid feeds occurs gradually (Adams et al., 2008). This slightly higher FS of individual animals normalised rapidly over time thanks to immediate oral rehydration therapy or habituation to solid feeds without showing significant aggravation of the health status. Higher FS can also be associated with the consumption of greater volumes of milk due to the undivided *(ad libitum)* feeding method (Anderson, 2013; Geiger et al., 2016). The results show that the acidified milk feeding did not strongly affect FS.

Growth Performance

The animal LW and LWG changes until 3 months of age are presented in Table 6.

Growth rate is one of the most important indices during the pre-weaning period as it reflects the overall outcome of management and husbandry (Breen et al., 2014; Van Amburgh, 2017). Regarding calf body LW changes before weaning, the only difference between the test groups was at 1 month of age when the average LW of experimental groups was significantly lower ($P < 0.05$) than the Control. All test group animals' LW at 1 month of age was similar to Schwarzkopf et al. (2019) who had LW results of late-weaned Holstein calves of around 60–68 kg at the 28 day age. Later - at the 60, 75, and 90 day age the average LW in all treatment groups of the present study was similar ($P > 0.05$). At weaning (75 day age), the LW of calves reached 107 ± 9.4 kg on average, which was sufficient to stop milk feeding.

Table 6. Growth performance of dairy calves fed different types of milk

Items	Age (days)	Growth performance						*p*-value
		EG-1		EG-2		Control		
		Mean	*SD*	*Mean*	*SD*	*Mean*	*SD*	
LW, kg	0	36.90	3.446	39.25	2.176	38.65	4.217	0.289
	30	60.79[a]	6.845	60.43[a]	6.901	66.69[b]	3.649	0.047
	60	87.44	9.791	88.22	6.892	89.95	8.523	0.797
	75 (weaning)	105.03	11.901	108.02	8.241	106.80	8.374	0.786
	90	127.34	11.474	130.32	11.317	133.04	12.124	0.556
LWG, g day^{-1}	0–30	796.3[ab]	216.7	706.2[a]	211.1	934.6[b]	75.0	0.028
	31–60	888.3[ab]	149.7	926.3[a]	104.9	775.3[b]	198.7	0.098
	61–75	1,172.7	345.6	1,320.0	462.0	1,123.3	369.5	0.520
	0–75 (pre-weaning)	908.4	147.1	916.9	103.3	908.7	77.1	0.982
	76–90 (post-weaning)	1,487.3	413	1,486.7	504.7	1,749.3	350.2	0.301

LW – body live weight; LWG – average daily live weight gain; [a, b] values with different superscript letters on the same line are different according to the independent samples *T-test* ($P < 0.05$).

At the 0–30 day period the LWG of EG-2 was lower than the Control group's ($P < 0.05$), but during the next 31–60 day period the results were opposite - the LWG of EG-2 was higher than the Control group's ($P < 0.05$). The initially lower LWG of EG-2 at 1 month of life may be explained by the lower intake of concentrates during the

15–28 day age (Table 7). The fluctuations of LWG are common and can also be caused by various other causes: infections and parasitism, nutrient deficiencies, environmental factors, as well as combinations thereof (Stratton-Phelps & Maas, 2015). Later - approaching the 2 month age (days 31–60), as well as 2 weeks before and after weaning (days 61–75, 76–90), the mean LWG of all animal groups was similar ($P > 0.05$). Overall, the LWG of the present study animals was typical to the high plane of milk feeding programmes with milk allowances ≥ 20% of the calf's birth weight, i.e., ≥ 750–1,000 g day^{-1} (Appleby et al., 2001; Jasper & Weary, 2002; Miller-Cushon, 2015). The LWG of the 2nd month of life of acidified milk-fed groups was similar to the Li et al. (2019) results of LWG 880–960 g day^{-1} of Holstein female calves (30–70 day age) fed reconstituted, reconstituted + acidified or acidified fresh milk for acidification using formic acid. Interestingly, Li et al. (2019) also observed positive effects of calves fed acidified liquid milk feed - animals exhibited greater withers height gain, as well as lower diarrhoea incidence, white blood cell and lymphocytes counts than calves fed reconstituted milk without acidification.

After weaning (76–90 day age) the LWG of all groups of the present study continued to increase, remaining similar ($P > 0.05$). The LWG of both acidified milk-fed groups was similar to the previously mentioned Sardoabi et al. (2021) study with calf LWG around 1,400–1,500 g day^{-1} at the 93 day age.

Regarding the effects of milk acidification on calves' growth and health, a good general animal health background of all treatment group animals in some respects did not allow the effectiveness of the milk acidification to be fully verified (e.g., the pasteurisation of milk and the cryptosporidial infection prophylaxis may have most likely helped to prevent diarrhoea). Coelho et al. (2020) concluded that acidification does not negatively affect animal growth in comparison to the refrigerated milk feeding; both diets resulted in calves with similar weaning weight; moreover acidification adjourns first diarrhoea and is a benefit itself in hot weather conditions when no refrigeration of milk is needed to maintain milk microbiological quality. A review carried out by Jones & Heinrichs (2014) on acidified milk feeding studies came to the conclusion that the health of calves is often neither improved nor deteriorated, and the results are not always consistent; they also concluded that there is little evidence that acidification itself affects the nutrients in milk or milk replacer or their utilisation by calves, calf performance, and LWG. A noteworthy discovery was made by Zhang et al. (2017) that calf health can also be influenced by the acidity level of milk. Decreasing the pH of milk replacer to a pH of 5.0–5.5 improved the digestive tract of pre-weaned calves (acidifier reduced the pH of digesta in the rumen, reticulum, and omasum, and was beneficial for the development of ruminal epithelium); in this study, hydrochloric acid was used to acidify the milk. However, a reduction to a pH of 4.5 had adverse effects on intestinal epithelium growth. Thus, it could indicate that the pH of the milk may also have affected the growth performance results of the present study - the lower LWG of EG-2 at 1 month of age could not only be related to the lower intake of concentrates, but also to the lower pH of the acidified milk, which could have a greater impact on young animals, as this trend later disappeared.

Feed Intake: ***Milk Intake*** **-** the total amounts of milk provided, consumed by calves and residual milk during the milk feeding diversification period (day 7–75) are shown in Fig. 1.

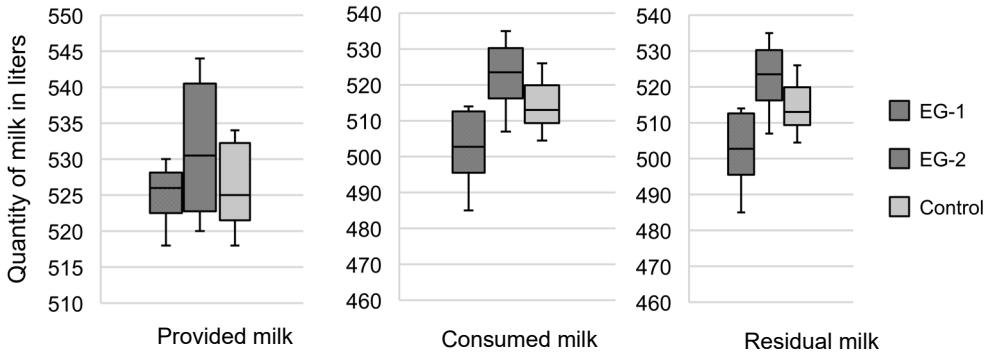

Figure 1. Summed up amounts of provided, eaten and uneaten milk of dairy calves fed different types of milk during the 7–75 day period until weaning.

In this study, none of the calves rejected the acidified milk. On the first days, some calves needed to get used to this taste of milk, but then willingly ate it. Regarding the provided milk during the 7–75 day period the total amount was similar in all treatment groups ($P > 0.05$): EG-1 (526 L; 522.0–528.0 L), EG-2 (530 L; 522.0–540.0 L), and the Control (525 L; 521.0–532.0 L) (medians, and minimum values-maximum values). The amounts of milk consumed by both experimental groups did not differ from the Control (513 L; 509.0–519.0 L) ($P > 0.05$). At the same time, there were significant differences between both experimental groups regarding the amounts of consumed and residual milk. The total consumed milk quantity during the 7–75 day period was significantly ($P < 0.05$) higher in the EG-2 group (523 L; 516.0–530.0 L) compared to EG-1 (502 L; 495.0–512 L) confirming that the method of feeding affects the quantity of the acidified milk consumed. It was already expected, because the milk feeding method of EG-2 was closer to the unrestricted method, and the time allowed for consuming it was longer. The largest amount of unconsumed milk was in group EG-1, being significantly higher than in both other groups ($P < 0.05$). The residual milk quantity for EG-1, EG-2, and the Control was 4.2% (22 L; 13.0–29.0 L), 1.3% (7 L; 5.0–11.0 L), and 1.9% (10 L; 8.0–13.0 L), respectively. Also regarding differences among individual animals, the biggest differences of leftover milk amounts were in the acidified milk-fed EG-1group. The milk was left over most particularly in the first month of life. Differences in appetite, satiety, attitude of individual animals towards the offered milk, its temperature or pH value can influence the milk intake. The results were most probably affected by the lower temperature of acidified milk fed to both experimental groups - 20–25 °C vs. 35–38 °C compared to the Control. Traditionally the milk is fed cool to slow the speed of intake and reduce the chance of gorge feeding (Anderson, 2013). Offering too cold acidified milk tends to reduce intake. There is also a practice to warm acidified milk to 38–40 °C (Coelho et al., 2020), yet milk must be fed immediately at this temperature because

holding acidified milk at temperatures above 24 °C will often cause curdling (Anderson, 2013; Jones & Heinrichs, 2014). Another reason for the differences of milk consumed among individuals can be related to milk acidity. Hill et al. (2013) observed that when feeding acidified milk replacer (MR) for *ad libitum* to Holstein calves, some animals completely rejected the MR at pH 4.2, and in general calves consumed more of the MR at pH 5.2 than pH 4.2. Choosing the right amount of acidifier is also important because not adding sufficient acid may affect the keeping quality of the product and lead to high bacteria counts or unpalatable spoiled milk, but reducing the pH by too much may limit palatability and cause calves to drink less milk. To promote the transition, it is advised to increase the acidity of the milk gradually (Jones & Heinrichs, 2014). Since milk is the main food for calves during the first month of life and summarising the above, it would be recommendable to extend the milk feeding time and increase its temperature to reduce the unconsumed milk quantity by younger calves in the case that the restricted (divided) feeding method is applied.

Feeding higher amounts of milk during later periods is not always considered positive regarding development of the rumen. Restricted feeding was introduced to encourage calves to eat concentrates as early as possible and thus to minimise costs for relatively expensive liquid feeds (Kertz et al., 1979). Which amount of milk should be fed is a complex issue that depends on the goals and the management capabilities of each individual farm (Lorenz et al., 2011).

Some aspects should also be noted regarding liquid feed composition changes via the addition of the acidifier being rich in lactose. Acidifier addition increased the milk lactose content by 0.15–0.19%. Thus, acidified milk-fed animals received 13–16 g of lactose per day more than the Control animals. As lactose is thought to promote the assimilation of calcium in the body and accelerate the process of ossification, it could have positive effects on young animal health. However, detailed research should be performed to confirm this assumption. The positive effect of lactose is explained by the fact that unhydrolysed lactose appears to be utilised as a prebiotic to support the growth of health-promoting gut flora, which is recognised as an enhancer of calcium absorption. The stimulating effect of lactose on calcium absorption was apparent in animal studies - both hydrolysed and unhydrolysed forms of lactose appeared to be positively involved in enhancing calcium absorption in mammals, but in humans such an effect is still controversial (Atkinson et al., 1957; Kwak et al., 2012). Lactose is the most important source of glucose for young calves and well metabolised in the body up to the age of 3–4 weeks (Latvietis, 2013). At a later age adult animals metabolise lactose relatively poorly - almost all of it is broken down by the microflora of the digestive tract, resulting in low pH. As the pH of the rumen decreases, systematic, prolonged feeding of lactose affects the intestinal microflora thereby impairing the digestion of other components of feed (Atkinson et al., 1957; Latvietis, 2013; Aschenbach et al., 2019). Thus, lactose ingestion has different effects depending on the age of the calves. Yet, as the increase in lactose content of the liquid feed due to the addition of acidifier in the present study can be considered as relatively small, its effects could be quite negligible and are unlikely to impair calf digestive tract health as evidenced by good FC, blood test, and LWG results of calves at 2 months of age.

Starter Intake - the summed up starter feed amounts eaten during different pre-weaning periods are presented in Fig. 2.

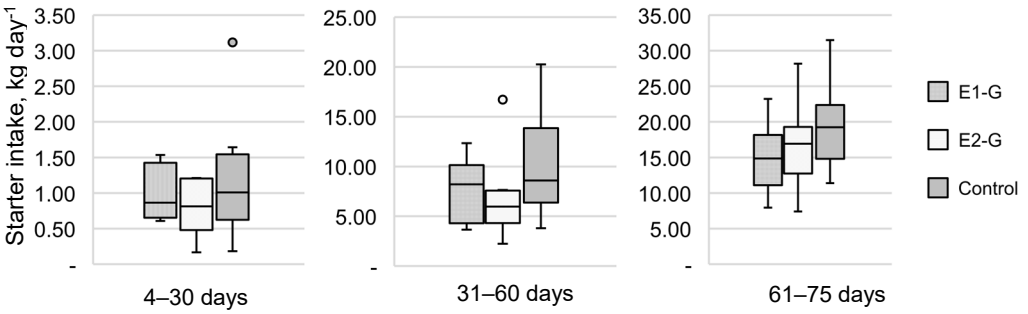

Figure 2. Summed up amounts of starter consumed by differently fed calves until weaning (data shown as median, Q1–Q3).

The differences between the groups on the summed up amounts of consumed starter feed during the 4–30, 31–60, and 61–75 day periods were not statistically significant ($P > 0.05$); however, when splitting the pre-weaning period into shorter periods, significant differences can be seen between the groups (Table 7).

Table 7. Starter intake of differently fed calf groups

Time period (days)	Starter intake, g day^{-1}						*p*-value
	EG-1		EG-2		Control		
	Median	Q1–Q3	Median	Q1–Q3	Median	Q1–Q3	
1–6	0	0–0	0	0–0	0	0–0	NA
7–14	0^{a}	0–23.3	15.0^{b}	0–43.3	3^{ab}	0–27.0	0.009
15–21	37.0^{a}	15.8–57.8	26.5^{b}	7.0–45.0	35.0^{ab}	11.0–73.0	0.044
22–28	64.0^{b}	50.5–94.5	25.0^{a}	11.0–57.8	42.5^{b}	21.0–124.0	0.001
29–35	89.5^{a}	47.3–130.0	58.5^{b}	27.5–103.0	83.5^{ab}	36.5–155.3	0.030
36–42	121.5^{a}	82.5–160.0	87.5^{a}	33.0–172.8	180.0^{b}	97.0–275.3	0.001
43–49	153.0^{a}	123.0–251.8	144.0^{a}	64.0–248.5	219.5^{b}	145.0–402.8	0.001
50–56	354.5^{b}	189.0–500.0	231.5^{a}	130.5–373.3	402.0^{b}	204.0–666.0	0.004
57–63	512.0^{ab}	368.7–882.0	514.5^{a}	357.3–746.3	719.0^{b}	445.3–1,031.0	0.016
64-74	1,046.0^{a}	733.5–1,384.8	1,113.5^{ab}	797.3–1,758.3	1,315.5^{b}	979.0–1,718.8	0.001

$^{a, b}$ Superscript values with different letters on the same line are different according to the Kruskal-Wallis h test with pair wise comparisons using Dunn's procedure ($P < 0.05$); NA – not applicable.

Results suggest that the starter quantity consumed by acidified milk-fed groups, especially EG-2, in quite a few periods was lower ($P < 0.05$) than by the calves of the Control group (36–49 and the 64–75 day periods for EG-1 and 15–63 day period for EG-2). Lower consumption of the starter by EG-2 animals can be associated with a larger amount of milk consumed by the undivided method allied to *ad libitum* feeding ($P < 0.05$) (Anderson, 2013; Akins, 2016; Geiger et al., 2016). Starter intake of the Leal et al. (2021) study calves receiving elevated milk replacer supply (8 L day^{-1}) was similar to our results: ~100 g day^{-1} at the 28 day age, and ~300–600 g day^{-1} at the 49–56 day age. Starter intake of 4 and 8 week old calves in the Coelho et al. (2020) study was higher - around 200–350 g day^{-1} and 750–900 g day^{-1}, respectively. In the present study, consumption of

concentrates increased noticeably after 1 month of age when the calf starts transition from a monogastric animal into a ruminant. Results confirm that starter intake is negligible in the first weeks of life, but after the first 3 weeks of life it increases and the calves start to grow rapidly (Lorenz et al., 2011; Schwarzkopf et al., 2019). Proper balancing of the quantities of milk provided with an amount of other kinds of feed is essential for the transition of calves from liquid to solid diets, because dry feeds are the stimulus for the development of the ruminal epithelium (Anderson, 2013; Akins, 2016; Diao et al., 2019; Sardoabi et al., 2021).

It should be mentioned that the starter for calves throughout the study was available *ad libitum*; besides that, since calves had free access to hay and were housed on straw, an uptake of hay and straw was also possible, the quantity of which was not measured. The amount of starter ingested is one of the key factors when determining the weaning age. Early consumption of starter dry matter is more important for systems in which the goal is early weaning and the lowest cost rearing programme (Davis & Drackley, 1998; Drackley, 2005). In the present study, weaning was finished at the age of 75 days when the starter intake of all treatment groups was above 1 kg day^{-1} in accordance with the starter producers' recommendations.

CONCLUSIONS

The results of the present study show that the whey-derived acidifier can be used in both - divided and undivided - milk feeding systems for pre-weaned calves' milk acidification having no adverse effects on animal health and growth. Various products containing highly concentrated acids are available on the market for the acidification of animal feed and must be diluted before being added to the milk feed. The advantage of the newly-developed product is that it is derived from food by-products, relatively easier to use and safer than other - more concentrated - products. Comparing acidified milk feeding methods, we concluded that the largest amount of unconsumed milk and the biggest differences among individual animals of leftover milk amounts were observed with the divided method for acidified-milk feeding (EG-1); therefore, it is recommendable to extend the feeding time and raise the acidified milk temperature to reduce the amount of leftover milk by younger calves. As for the second - the undivided method - it saves the time and labour required for feeding, as well as reduces the loss of milk; yet consumption of the starter was lower. As results observed regarding growth performance and health indices of all dietary treatment groups of calves were similar, we could anticipate that the acidification benefits would be greater when providing unpasteurised milk, or during the hottest weather when the risks of milk spoilage are greater, as previously concluded by other researchers (Jones & Heinrichs, 2014; Coelho et al., 2020). Therefore an in-depth study of the tested acidifier efficiency in other production, housing, and more extreme conditions would be useful.

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