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# The type of lipid supplement has crucial implications for forage particle size in calf starter diets

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## Abstract

**Background** Forage inclusion in starters of young dairy calves has become an acceptable strategy in the last decade. To compensate for the lower energy provided by forage, concurrent lipid supplementation can be proposed. However, ruminal microbial activity and forage digestibility may be decreased by lipid supplementation. We hypothesized that the composite effect of forage and lipid supplements may be dependent on forage particle size and the type of lipid supplement. Therefore, we evaluated the effect of long (LP; geometric mean, 4.97 mm) vs. short alfalfa hay particle sizes (SP; geometric mean, 1.26 mm) with either soybean oil (SBO) or palm fatty acids (PLF) as lipid source in a 2 × 2 factorial design with treatments SP-SBO, SP-PLF, LP-SBO, and LP-PLF. Treatments ( $n = 13$  with 6 males and 7 females each) were offered to Holstein calves (3 days old) with equal amounts of lipid (25 g/kg DM) throughout the experimental period. The milk offering scheme (d 1 to 53) was equal for all groups. Data collection continued until 20 d post-weaning.

**Results** Interaction between forage particle size and lipid supplement was significant for the following readouts: the highest and lowest starter intakes during the pre-weaning period occurred in LP-PLF and LP-SBO, respectively. This was associated with similarly contrasting changes in average daily gain (ADG) during the post-weaning period, body weight at the end of experiment, withers height, digestibility of organic matter and neutral detergent fiber, and blood serum concentrations of glucose, beta-hydroxybutyrate, and insulin during the pre-weaning period. During both pre- and post-weaning periods, the highest and lowest urinary excretion of allantoin and total purine derivatives, representing microbial protein synthesis, were observed in LP-PLF and LP-SBO, respectively, indicating that those diets were most and least favorable for rumen development. Irrespective of forage particle size, supplemental SBO vs. PLF increased serum malondialdehyde as an oxidative stress indicator across periods, increased blood urea nitrogen and feed efficiency in the pre-weaning period, and reduced hip height during the post-weaning period.

**Conclusions** It can be concluded that feeding a rumen-inert, mostly saturated fatty acid source with alfalfa hay as long particle size is recommended with view on performance, whereas a combination soybean oil rich in unsaturated fatty acids should not be provided to milk-fed Holstein calves together with long particle forage. Feeding soybean oil and alfalfa hay as long particles is not advisable mainly due to lower starter consumption and impaired

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development of ruminal function. If dietary supplementation of soybean oil is applied, incorporation of forage as small particles should be preferred to support rumen development.

**Keywords** Forage particle size, Lipid supplement, Oxidative stress, Ruminal functional development

## Introduction

Forage inclusion in the starter feed of young dairy calves has been considered as a favorable feeding strategy in recent years. This feeding strategy aims to stabilize ruminal pH and to improve ruminal fermentation while also preventing hyperkeratinization and clumping of ruminal papillae [1]. In addition to ruminal effects, recent studies indicated that forage inclusion in starter feeds of young calves has potential to maintain proper metabolic and hormonal status with improvement of the somatotrophic axis [2]. Contrary to these considerations, some worry that forage may displace concentrate intake and shift ruminal fermentation towards acetate rather than butyrate production, thus, delay ruminal papillae development in young ruminants [3]. Further arguments against starter provision with high forage content to young ruminants are expectations of reduced starter intake and body weight gain [4], reduced ruminal wall development and impaired nutrient transport and metabolism [5].

Favorable and unfavorable impacts of feeding forages in starter feed of calves on growth performance and rumen fermentation may be dependent on the source of forage, forage feeding level, method of processing, and its chemical composition [6, 7]. Furthermore, forage particle size (FPS) can also be an important issue for the response of young calves to forage inclusion in starters [7, 8]. As stated in NRC (2001), ruminants need dietary forage with an adequate particle size to enhance fiber digestion and maintain their health [9]. Although discussion is made extensively on forage intake in young dairy calves; however, no clear recommendation is available for FPS in the recommendations of the National Academies of Science, Engineering, and Medicine [10]. A shorter than optimum FPS may result in low dry-matter (DM) intake, decreased fiber digestibility, and impaired health status. The reciprocal with higher than recommended FPS may provoke feed sorting, excessive time spent for re-chewing, higher retention time of digesta and reduced fractional passage kinetics of particulates. Limited documents are available with respect to the effect of FPS on dairy calves' performance.

Regardless of the particle size of forage incorporated in starter feeds, lower energy content of forage compared to concentrate can provide less energy in forage-based starter diets in comparison with all-concentrate starter feeds [4, 11]. The lower energy supplied through

forage compared to concentrate may be considered as an obstacle in accelerated growth programs with early weaning in commercial dairy farms. Lipid supplementation can be applied as a strategy in forage-containing starters to compensate the energy deficit [12, 13]. In fact, it can be postulated that concurrent feeding of forage and lipid may be a practical way to support both stable ruminal conditions and high performance of young calves. The biological activities of lipids extend beyond serving as an energy source and maintaining normal biological functions; however, there is limited understanding of how they are utilized by developing dairy calves. In recent studies, oil supplementation to growing lambs' diet improved ruminal health and development, feeding behavior and animal performance [14], as well as ruminal epithelial function and metabolism [15]. A better understanding of lipid metabolism and the impacts of supplementation of dietary fats or manipulations of energy sources may offer new ways to enhance development and improve performance of growing replacement heifers through nutritional strategies [16]. A negative interaction may be present between fiber and lipids in ruminants, which can be due to coating effects of lipids on fiber digestion and toxic effects on ruminal micro-organisms due to compounds produced by lipids metabolism [17]. The negative effect of lipids on fiber digestion may be more of a concern in dairy calves compared to mature ruminants due to their less developed microbial community. Some previous studies addressed the interaction effect of forage source and lipid supplement on performance of dairy calves [11, 13]; however, the combined effects of forage particle size and lipid supplement need to be evaluated in dairy calves. In addition, as stated in previous studies [12, 14, 16], lipid supplement may have different impacts from the oxidative stress perspective, which can eventually influence whole animal body metabolism. The interactive effect of FPS with different lipid supplement on the oxidative stress of young calves also needs to be evaluated in dairy calves. Therefore, the aim of the current study was to evaluate the interaction effect between different forage particle sizes (geometric mean particle lengths equal to 1.26 mm as SP vs. 4.97 mm as LP) and supplemental lipid source (SBO vs. PLF) on growth performance, blood metabolites, blood insulin concentration, oxidative stress indicators, and urinary purine derivatives in young dairy calves.

## Materials and methods

### Calves and management

The present study was conducted on a commercial dairy farm located in the Qazvin province, Iran. The experimental design, management protocols, and procedures were approved by the Animal Care Committee at the University of Zanjan, Iran (ID 1353). Fifty-two, healthy Holstein dairy calves (24 males and 28 females;  $41.1 \pm 2.9$  kg BW at birth) were selected at 3 days of age and randomly allocated to 4 groups (13 calves per treatment; 6 males and 7 females, each) in a  $2 \times 2$  factorial arrangement. Calves were housed in  $1.3 \text{ m} \times 2.4 \text{ m}$  individual pens. The floor of each pen was bedded with sand that was renewed every 24 h. Each calf was fed 5 L of colostrum during the first 12 h of life (2.5 L within 1.5 h after birth and 2.5 L in a second feeding) which was continued during the first 2 d of life. Colostrum quality was tested using a digital Brix refractometer (PAL-1, Atago Co., Ltd., Bellevue, WA, USA) and only colostrum with Brix scale greater than 22 was used. The experiment started on d 3. Calves were fed 4.5, 7.5, 2.5 and 1.5 L/d of whole milk from d 3 to 10, 11 to 40, 41 to 51 and 52 to 53, respectively. Fat, protein, lactose and total solid of milk samples were measured weekly by using an infrared spectrophotometer (FOSS Milk-O-Scan, FOSS Electric, Hillerod, Denmark). The average composition of consumed milk was  $31.2 \pm 0.7$ ,  $31.3 \pm 0.3$ ,  $49.2 \pm 0.3$  and  $118 \pm 0.4$  g/kg of fat, protein, lactose and total solid, respectively. All calves were weaned at 53 days of age but maintained on the experimental diets until 73 days of age.

### Experimental starter diets

Experimental diets were formulated according to NRC 2001 [9] recommendations to meet nutrient requirements, but differed in alfalfa hay particle sizes and lipid source according to treatment. Four experimental diets were: 1) small particles of alfalfa hay with soybean oil (SP-SBO); 2) small particles of alfalfa hay with palm fatty acids (SP-PLF); 3) long particles of alfalfa hay with soybean oil (LP-SBO); and 4) long particles of alfalfa hay with palm fatty acids (LP-PLF). The feed ingredients and the chemical composition of experimental starter diets are given in Table 1. A Penn State particle separator (Nasco, Fort Atkinson, WI, USA) equipped with 3 sieves (19, 8, and 1.18 mm) and a bottom pan were used to separate the differently chopped alfalfa hay for particle size analysis into long (> 19 mm), medium (8–19 mm), short (1.18–8 mm), and fine (< 1.18 mm) fractions [18]. The resulting geometric mean of particle size (GMPL) for the two alfalfa hay feed ingredients was calculated as described by the American Society of Agricultural Engineers [19]. These were 1.26 and 4.97 mm for chopped alfalfa hay with small and long particle sizes, respectively. The concentrate feed

**Table 1** Experimental starter feed ingredients and chemical composition

Item	Treatments <sup>1</sup>			
	SP		LP	
	SBO	PLF	SBO	PLF
Ingredients, g/kg of DM				
Alfalfa hay (chopped, GMPL <sup>2</sup> : 1.26 mm)	100	100	0	0
Alfalfa hay (chopped, GMPL: 4.97 mm)	0	0	100	100
Barley grain, ground	140	140	140	140
Corn grain (coarsely ground)	420	420	420	420
Soybean meal	270	270	270	270
Soybean oil	25	0	25	0
Palm fatty acids	0	25	0	25
Calcium carbonate	8	8	8	8
Sodium bicarbonate	7	7	7	7
Di-calcium phosphate	6	6	6	6
Salt	4	4	4	4
Vitamin and mineral mix <sup>3</sup>	20	20	20	20
Chemical composition, g/kg of DM, unless stated otherwise				
Metabolizable energy <sup>4</sup> , Mcal/kg	2.94	2.94	2.94	2.94
CP	208	208	208	208
NDF	184	184	184	184
Ether extract (EE)	56.3	56.3	56.3	56.3
Non-fiber carbohydrate <sup>5</sup>	501	501	501	501
Ca	84	84	84	84
P	45	45	45	45

<sup>1</sup> Treatments: alfalfa hay provided as small particle size supplemented with soybean oil (SP-SBO); alfalfa hay provided as small particle size supplemented with palm fatty acids (SP-PLF); alfalfa hay provided as long particle size supplemented with soybean oil (LP-SBO); alfalfa hay provided as long particle size supplemented with palm fatty acids (LP-PLF)

<sup>2</sup> GMPL: Geometric mean of particle size

<sup>3</sup> Containing per kg: Vit A (IU) = 1,000,000, Vit D (IU) = 110,000, Vit E (IU) = 1,100 Ca (g) = 90, P (g) = 25, Mg (g) = 30, Zn (mg) = 1,700, Cu (mg) = 540, I (mg) = 110, Co (mg) = 80, Mn (mg) = 1,900, Se (mg) = 140

<sup>4</sup> Calculated according to NRC (2001) [9]

<sup>5</sup> Non-fibre carbohydrate was calculated as DM – (NDF + CP + EE + ash) (NRC, 2001) [9]

inclusive of lipid source was mixed well with forages and the diet was offered as total mixed ration throughout the study. The SBO was sourced from Naz Industrial (Vegetable Oil Co., Isfahan, Iran) and had the following fatty acid composition: C16:0, C18:0, C18:1, C18:2, C18:3, and other fatty acids equal to 12.1%, 5.2%, 21.8%, 51.2%, 8.1%, and 1.6%, respectively. The composition of PLF (rumen-inert; Energizer RP-10, IFFCO, Johor, Malaysia) was C12:0, C14:0, C16:0, C18:0, C18:1, and other fatty acids equal to 2.3%, 4.2%, 86.0%, 2.0%, 4.1%, and 1.4%, respectively. The starters were fed ad libitum from d 3 onwards in an amount that permitted at least 10%orts. The calves had free access to water throughout the experimental period.

### Intake, feed efficiency, digestibility, and fecal score

Experimental diets were offered once daily at 0800 h and orts were collected and recorded the following morning (0730 h). The starter DM intake was calculated as the difference of offered starter feed and collected orts for each calf. The BW of calves was measured on d 3 (initial day of experiment) and then every 10 d during the study; BW recording was performed before feeding to avoid bias by gastrointestinal fill. Average daily gain (ADG) and feed efficiency (FE: kg of BW gain divided by kg of total DM intake; the latter being the sum of milk DM and starter DM) were calculated before (d 3–53) and after (d 54–73) weaning, and for the entire period (d 3–73). Samples of experimental diets and orts were dried at 60 °C for 48 h in a convection oven every 14 d. To determine chemical composition, subsamples of dried experimental starter diets and their orts were mixed thoroughly and ground in a mill (Ogaw Seiki CO., Ltd., Tokyo, Japan) to pass a 1-mm screen. Experimental diets and their orts were analyzed for DM (method No. 2001.12), ash (method No. 942.05), CP (method No. 991.20), and ether extract (EE; method No. 920.39) according to standard procedures [20]. To determine neutral detergent fiber (NDF), samples of experimental diets were analyzed by applying a heat-stable alpha-amylase without sodium sulfite addition and with correction for residual ash and N [21]. Apparent total tract digestibility of nutrients was measured during four congestive days at post-weaning period (d 69–72) using acid insoluble ash as an internal marker [22]. Fecal scoring was performed according to Ghorbani et al. [23] as 1 = firm, 2 = soft, 3 = soft and running, and 4 = watery.

### Skeletal growth parameters

Skeletal growth parameters of all calves, including withers height, body barrel, hip height, heart girth, and body length were measured at the beginning of experiment (d 3), at weaning (d 53) and at the last day of experiment (d 73) as described for young dairy calves [24].

### Blood metabolites, insulin, and oxidative stress indicators

Blood samples were obtained without preservative on d 35 (before weaning) and 71 (after weaning) from the jugular vein at 2 h after the morning meal and allowed to clot. Immediately after centrifugation of clotted samples at  $3,000 \times g$  for 15 min at 4 °C, serum was separated and stored at –20 °C until subsequent measurements. Serum concentrations of glucose, urea-N (BUN), triglycerides, cholesterol, and total protein were determined using autoanalyzer (UNICCO, 2100; Zistchemi Co., Tehran, Iran) with appropriate analytical kits (Pars-Azemun Co., Ltd., Karaj, Iran). Serum beta-hydroxybutyrate (BHB) was measured using a commercial kit (Abbott Diabetes

Care Ltd.) and insulin concentration was measured using Insulin AccuBind ELISA (MonobindInc, Lake Forest, CA, USA) according to manufacturers' instructions.

Activities of glutathione peroxidase (GPx), superoxide dismutase (SOD), and concentration of malondialdehyde (MDA) in blood were measured as oxidative stress indicators using a plate reader (DANA 3200, Garny, Iran). Activity of GPx was evaluated by Paglia and Valentine's method [25] in whole blood, using RANSEL Kit (Randox, UK). In this kit, GPx catalyses the oxidation of glutathione cumene hydroperoxide. SOD activity was measured in haemolysate by an iodophenyl nitrophenol phenyltetrazolium chloride modified method (RANSOD Kit, Randox, UK). This method employs xanthine and xanthine oxidase to generate superoxide radicals which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride to form a red formazan dye. SOD activity was then measured by the degree of inhibition of this reaction [26]. Measurement of MDA was performed by the thiobarbituric acid method, for which reactive substances were measured with thiobarbituric acid as peroxidation index. The basis of this measurement is the MDA reaction with thiobarbituric acid, which results in the formation of pink colour, and is calculated by measuring the intensity of the color produced at 520 nm [27].

### Urinary purine derivatives and microbial protein synthesis

For estimating microbial protein synthesis (MPS) in the rumen, the spot sampling technique was used, which is based on purine derivatives (PD) excretion in urine. Total urine volume was estimated as  $BW \times 26.8 / \text{creatinine concentration (mg/L)}$  in dairy calves less than 4 months of age, as recently reported by Dennis et al. [28]. Urine spot samples (approximately 10 mL) were collected at four consecutive days during the pre-weaning period (41–44 d) and post-weaning period (66–69 d) between 0900 and 1100 h and 1500 and 1700 h at the time that calves had spontaneous urination. A sub-sample of 5 mL of each sample was diluted immediately with 45 mL of 0.036 N sulfuric acid and stored at –20 °C for analysis. Sub-samples were pooled per animal and sampling period before analysis. The analysis was performed as previously reported [29]. In brief, the concentrations of creatinine (Kit No. 555-A; Sigma Chem. Co., St. Louis, MO, USA) and uric acid (Kit No. 685–50 Sigma Chem. Co.) were measured using spectrophotometer (UV-2600i, Shimadzu, Japan) in accordance with the manufacturer's instructions. For allantoin determination the high-performance liquid chromatography as model 10 A high-performance liquid chromatograph (Shimadzu, Kyoto, Japan) equipped with a UV spectrophotometric detector set at 220 nm was used [30]. The daily endogenous PD was considered to be  $705 \mu\text{mol/kg}$  of  $BW^{0.75}$  according



to previous suggestions in dairy calves [31]. To prevent overestimation of MPS due to PD contained in the consumed milk, 280  $\mu\text{mol PD/L}$  of milk were subtracted from the total PD during the pre-weaning period [32]. For post-weaning calves, no correction was made, and it was assumed that the total PD via urine minus endogenous PD was of microbial origin, as reported in a previous study of post-weaning calves [30]. The MPS in the rumen of calves was then calculated from daily urinary PD output using the following equation described by Chen and Gomes [33]: Microbial N (g N/d) =  $X$  (mmol/d)  $\times$  70 / (0.116  $\times$  0.83  $\times$  1,000), where  $X$  is the microbial purine uptake (mmol/d), 70 is the N content of the purine coefficient (mg N/mmol), 0.116 is the ratio of purine N to total N in mixed rumen microbiota (which is 11.6:100), and 0.83 is the average digestibility of microbial purines [33].

### Statistical analysis

All data were analyzed using PROC MIXED of SAS (version 9.1; SAS Institute, Cary, NC, USA). Individual calf was used as experimental unit. When repeated measurements were available within a period (starter DM intake, ADG, and FE), data were additionally treated as repeated measures per period. Data at each time point/period were tested using the following model:  $Y_{ijk} = \mu + FPS_i + Fat_j + [FPS \times Fat]_{ij} + \beta [X_i - \bar{X}] + \varepsilon_{ijk}$  where  $Y_{ijk}$  is the dependent variable;  $\mu$  is the overall mean;  $FPS_i$  is the effect of alfalfa hay particle size (small vs. long particle sizes);  $Fat_j$  is the effect of lipid source (SBO vs. PLF); and  $[FPS \times Fat]_{jk}$  is the two-way interaction effect of alfalfa hay particle size and lipid source;  $\beta [X_i - \bar{X}]$  is considered as covariate and  $\varepsilon_{ijk}$  is the overall error term. When data were available at different time points/periods (all data except milk intake, total dry matter intake and nutrient digestibility), the effects across time points/periods were also tested in a second step using the model  $Y_{ijkl} = \mu + FPS_i + Fat_j + [FPS \times Fat]_{ij} + T_k + [FPS \times T]_{ik} + [Fat \times T]_{jk} + [FPS \times Fat \times T]_{ijk} + \beta [X_i - \bar{X}] + \varepsilon_{ijkl}$  where  $T_k$  is the effect of time (pre-weaning vs. post-weaning period or the three time points of BW measurement); and  $[FPS \times T]_{ij}$ ,  $[Fat \times T]_{jk}$ , and  $[FPS \times Fat \times T]_{ijk}$  are the two- and three-way interaction effects of time with the factors alfalfa hay particle size and lipid source. The effects of sex were also tested, including all two- and three-way interactions with the factor sex. Because these were not significant for any of the variables tested, except for a tendency regarding withers height ( $P=0.09$ ), sex was completely excluded from the model. An autoregressive (order 1) covariance structure was chosen according to the Akaike and Bayesian information criteria. Data were analyzed for three periods including before weaning (d 3–53), after weaning (d 54–73) and the entire period (d 3–73). The

initial measurements (d 3) were included as a covariate for the analysis of BW and skeletal growth parameters at weaning and at the end of experiment. The fecal score was square-root transformed for better homogeneity of the distribution of residuals. Tukey's multiple range tests were used to determine the differences among experimental diets means. Results were considered to be significant when  $P \leq 0.05$ , and a tendency was considered when  $0.05 < P \leq 0.10$ . All data are reported as least square means with pooled standard error of mean (SEM).

## Results

### Starter intake, gain and efficiency, fecal score, and nutrient digestibility

During the pre-weaning period, starter intake was lower for SBO compared to PLF ( $P=0.001$ ) and, additionally, an interaction was found for  $FPS \times$  lipid supplement with the highest starter intake found in LP-PLF and lowest starter intake found in LP-SBO ( $P=0.048$ ; Table 2). No effect of FPS, lipid supplement, or their interaction was detected during the post-weaning period. Milk intake did not differ among experimental treatments; however, because of lower starter intake in calves fed SBO diets, TDMI (milk DM + starter DM) was lower in these calves compared to calves receiving PLF diets ( $P=0.001$ ). Post-weaning and over the entire period, ADG was affected by lipid supplement ( $P=0.012$  and  $P=0.008$ , respectively) with an interaction between  $FPS \times$  lipid supplement ( $P=0.037$  and  $P=0.026$ , respectively), resulting in highest ADG in LP-PLF and lowest ADG in LP-SBO. In accordance with the changes for ADG, BW at weaning and final BW were affected by lipid supplement ( $P=0.003$  and  $P=0.001$ , respectively) with an interaction between  $FPS \times$  lipid supplement over the entire period ( $P=0.014$ ), leading to highest final BW in LP-PLF and lowest final BW in LP-SBO. Feed efficiency was lower during the pre-weaning period ( $P=0.048$ ) and entire period of experiment ( $P=0.029$ ) in calves that received SBO compared to PLF.

Fecal score was higher (i.e., calves had looser feces) when calves were fed SBO in comparison to PLF during pre-weaning ( $P=0.001$ ) and the entire experiment ( $P=0.004$ ; Table 2).

Regarding the time effect, starter feed intake, TDMI, ADG, and BW all increased from the pre-weaning to the post-weaning period ( $P=0.003$ , 0.009, 0.001, and 0.004, respectively) with interactions for  $FPS \times$  lipid  $\times$  time ( $P=0.007$ , 0.005, 0.002, and 0.003, respectively) that largely reflected the interactions observed in individual periods. Feed efficiency and fecal scores decreased from pre-weaning to post-weaning ( $P=0.006$  and 0.001, respectively) with an  $FPS \times$  lipid  $\times$  time interaction ( $P=0.001$  and 0.008, respectively) indicating that despite a missing

**Table 2** Least square means for starter intake, average daily gain, feed efficiency, and nutrient digestibility in dairy calves fed different alfalfa particle sizes (small particles vs. long particles) and lipid supplements (soybean oil and palm fatty acids) ( $n=13$  calves per treatment)

Item	Treatments <sup>1</sup>				SEM	P-value <sup>2</sup>							
	SP		LP			FPS	Lipid	FPS×Lipid	T	FPS×T	Lipid×T	FPS×Lipid×T	
	SBO	PLF	SBO	PLF									
Starter feed intake, g/d													
Pre-weaning (d 3–53)	435 <sup>b,BC</sup>	573 <sup>ab,BC</sup>	336 <sup>cC</sup>	606 <sup>a,B</sup>	34.7	0.33	0.001	0.048					
Post-weaning (d 54–73)	2,265 <sup>A</sup>	2,259 <sup>A</sup>	2,307 <sup>A</sup>	2,438 <sup>A</sup>	90.1	0.14	0.33	0.31					
Entire period (d 3–73)	958	1,056	899	1,142	97.2	0.88	0.72	0.44	0.003	0.006	0.002	0.007	
Milk intake, g/d	676	673	671	675	23.4	0.99	0.98	0.97					
Total dry matter intake (milk + starter), g/d	1,112	1,249	1,011	1,281	41.3	0.41	0.001	0.11					
Average daily gain, g/d													
Pre-weaning (d 3–53)	451 <sup>C</sup>	540 <sup>BC</sup>	436 <sup>C</sup>	624 <sup>B</sup>	34.2	0.25	0.004	0.093					
Post-weaning (d 54–73)	728 <sup>b,AB</sup>	778 <sup>b,AB</sup>	658 <sup>c,B</sup>	839 <sup>a,A</sup>	38.8	0.89	0.012	0.037					
Entire period (d 3–73)	530 <sup>D</sup>	608 <sup>b</sup>	499 <sup>c</sup>	685 <sup>a</sup>	29.6	0.37	0.008	0.026	0.001	0.003	0.002	0.002	
Body weight													
Initial (d 3)	41.1 <sup>F</sup>	40.9 <sup>F</sup>	41.7 <sup>F</sup>	41.2 <sup>F</sup>	1.30	0.66	0.75	0.91					
Weaning (d 53)	63.2 <sup>E</sup>	67.4 <sup>E</sup>	63.5 <sup>E</sup>	72.4 <sup>DE</sup>	1.81	0.12	0.003	0.072					
Final (d 73)	78.1 <sup>b,BC</sup>	82.9 <sup>b,B</sup>	76.2 <sup>c,CD</sup>	89.2 <sup>a,A</sup>	1.96	0.14	0.001	0.014	0.004	0.009	0.005	0.003	
Feed efficiency <sup>3</sup>													
Pre-weaning (d 3–53)	0.402 <sup>AB</sup>	0.431 <sup>AB</sup>	0.429 <sup>AB</sup>	0.486 <sup>A</sup>	0.0357	0.10	0.047	0.51					
Post-weaning (d 54–73)	0.338 <sup>BC</sup>	0.354 <sup>B</sup>	0.302 <sup>C</sup>	0.339 <sup>BC</sup>	0.0413	0.22	0.202	0.62					
Entire period (d 3–73)	0.384	0.409	0.393	0.445	0.0449	0.21	0.029	0.45	0.006	0.046	0.001	0.001	
Fecal score													
Pre-weaning (d 3–53)	2.0 <sup>A</sup>	1.6 <sup>B</sup>	1.8 <sup>AB</sup>	1.6 <sup>AB</sup>	0.54	0.13	0.001	0.43					
Post-weaning (d 54–73)	1.3 <sup>C</sup>	1.3 <sup>C</sup>	1.4 <sup>BC</sup>	1.4 <sup>BC</sup>	0.39	0.47	0.97	0.85					
Entire period (d 3–73)	1.9	1.5	1.8	1.5	0.48	0.32	0.004	0.45	0.001	0.081	0.023	0.008	
Nutrient digestibility, %													
Organic matter	70.4 <sup>b</sup>	71.3 <sup>ab</sup>	66.8 <sup>c</sup>	75.9 <sup>a</sup>	1.83	0.72	0.026	0.006					
Ether extract	84.6	86.0	85.5	86.1	1.31	0.71	0.38	0.66					
Neutral detergent fiber	42.4 <sup>ab</sup>	39.1 <sup>b</sup>	32.5 <sup>c</sup>	49.9 <sup>a</sup>	1.84	0.90	0.034	0.042					
Crude protein	56.6	62.9	68.5	65.3	1.36	0.46	0.20	0.97					

<sup>1</sup> Treatments: alfalfa hay provided as small particle size supplemented with soybean oil (SP-SBO); alfalfa hay provided as small particle size supplemented with palm fatty acids (SP-PLF); alfalfa hay provided as long particle size supplemented with soybean oil (LP-SBO); alfalfa hay provided as long particle size supplemented with palm fatty acids (LP-PLF)

<sup>2</sup> Statistical comparisons: Forage particle size (FPS) = small versus long particle sizes; Lipid = soybean oil versus palm fatty acids; FPS×Lipid = interaction between alfalfa hay particle sizes and supplemental lipid; T = time; FPS×T = forage particle size×time; Lipid×T = lipid source×time; FPS×Lipid×T = forage particle size×lipid source×time

<sup>3</sup> kg of body weight/kg of total dry matter intake

<sup>a–c</sup> Different small letters indicate differences of a variable within one period (interaction FPS×Lipid;  $P \leq 0.05$ )

<sup>A–F</sup> Different capital letters indicate differences of a variable across periods (interaction FPS×Lipid×T;  $P \leq 0.05$ )

two-way interaction of FPS×lipid, these factors separated differently when additionally considering the growing stage of the animal. For feed efficiency, the highest and lowest efficiencies were observed in LP-PLF pre-weaning and LP-SBO post-weaning, respectively. For fecal score, the highest value was observed in SP-SBO pre-weaning; however, this value improved to a commonly low level observed across treatments in the post-weaning period.

Digestibility of ether extract was not influenced by FPS. However, the digestibility of OM ( $P=0.026$ ) and NDF ( $P=0.034$ ) were lower in calves fed SBO compared to calves receiving PLF with an interaction showing that digestibility of OM ( $P=0.006$ ) and NDF ( $P=0.042$ ) was highest in LP-PLF and lowest in LP-SBO (Table 2).

### Structural growth indices

Withers height at weaning and at the end of experiment were greatest in LP-PLF and lowest in LP-SBO as supported by FPS×lipid supplement interactions ( $P=0.001$  and  $0.003$ , respectively; Table 3). Body barrel was greater in calves fed long particles compared to calves fed short particles of alfalfa hay (post-weaning;  $P=0.039$ ). Feeding SBO to calves resulted in a tendency for lower hip height at weaning ( $P=0.075$ ) and lower hip height at the final day of experiment ( $P=0.028$ ). No effect of FPS, lipid supplement, or their interaction was found for body length, heart girth, and hip height.

As expectable from the accelerated BW gain in the post-weaning period described in the previous

sub-section, all structural growth indices increased from the pre-weaning to the post-weaning period (all  $P\leq 0.01$ ); however, without any two- or three-way interactions for the factor time.

### Blood metabolites, insulin, and oxidative stress indicators

The blood serum concentrations of glucose ( $P=0.029$ ) and BHB ( $P=0.077$ ) were or tended to be affected by lipid supplement during the pre-weaning period with FPS×lipid supplement interactions ( $P=0.003$  and  $0.009$ , respectively), showing that their concentrations were highest in LP-PLF and lowest in LP-SBO. During the post-weaning period, the serum concentration of glucose tended to be lower ( $P=0.056$ ) and the concentration

**Table 3** Least square means for structural growth indicators (cm) in dairy calves fed different alfalfa particle size (small particles vs. long particles) and lipid sources (soybean oil and palm fat) ( $n=13$  calves per treatment)

Item	Treatments <sup>1</sup>				SEM	P-value <sup>2</sup>				T	FPS×T	Lipid×T	FPS×Lipid×T
	SP		LP			FPS	Lipid	FPS×Lipid					
	SBO	PLF	SBO	PLF									
Body barrel													
d 53	112	113	111	112	5.4	0.44	0.78	0.74					
d 73	114	115	118	119	6.1	0.039	0.19	0.55					
Overall	113	114	114	115	5.8	0.048	0.67	0.71	0.007	0.54	0.093	0.69	
Body length													
d 53	60.0	59.1	59.8	60.2	0.84	0.99	0.98	0.82					
d 73	62.8	63.3	64.2	63.8	0.91	0.55	0.91	0.66					
Overall	61.7	61.1	62.2	61.8	0.86	0.50	0.62	0.89	0.003	0.74	0.77	0.44	
Heart girth													
d 53	98.6	97.2	97.5	99.2	1.19	0.62	0.91	0.14					
d 73	105	104	103	107	1.41	0.95	0.26	0.31					
Overall	101	101	100	102	1.50	0.78	0.34	0.064	0.001	0.71	0.42	0.94	
Withers height													
d 53	94.9 <sup>ab</sup>	92.2 <sup>b</sup>	90.7 <sup>c</sup>	96.8 <sup>a</sup>	1.52	0.84	0.083	0.001					
d 73	99.0 <sup>ab</sup>	96.6 <sup>b</sup>	96.1 <sup>b</sup>	100 <sup>a</sup>	1.93	0.47	0.37	0.003					
Overall	97.0 <sup>b</sup>	94.4 <sup>bc</sup>	93.6 <sup>c</sup>	98.7 <sup>a</sup>	1.85	0.52	0.05	0.018	0.009	0.73	0.48	0.34	
Hip height													
d 53	90.6	91.2	90.9	93.0	1.13	0.12	0.075	0.22					
d 73	94.6	96.5	95.8	96.3	1.28	0.33	0.028	0.21					
Overall	92.7	93.8	93.3	94.6	1.30	0.052	0.019	0.85	0.004	0.48	1.00	0.073	
Hip width													
d 53	19.0	18.3	18.4	18.6	0.78	0.83	0.63	0.44					
d 73	20.1	19.8	20.7	20.3	0.80	0.30	0.51	0.79					
Overall	19.5	19.0	19.6	19.4	0.73	0.53	0.41	0.73	0.001	0.36	0.88	0.47	

<sup>1</sup> Treatments: alfalfa hay provided as small particle size supplemented with soybean oil (SP-SBO); alfalfa hay provided as small particle size supplemented with palm fat (SP-PLF); alfalfa hay provided as long particle sizes supplemented with soybean oil (LP-SBO); alfalfa hay provided as long particle size supplemented with palm fat (LP-PLF)

<sup>2</sup> Statistical comparisons: Forage particle size (FPS) = small versus long particle sizes; Lipid = soybean oil versus palm fatty acids; FPS × Lipid = interaction between alfalfa hay particle sizes and supplemental lipid; T = time; FPS × T = forage particle size × time; Lipid × T = lipid source × time; FPS × Lipid × T = forage particle size × lipid source × time

<sup>a-c</sup> Different small letters indicate differences of a variable within one period (interaction FPS × Lipid;  $P\leq 0.05$ )

of BHB were lower ( $P=0.028$ ) in calves receiving SBO (Table 4). Blood urea nitrogen was lower in calves fed PLF compared to SBO during the pre-weaning period ( $P=0.035$ ). Other blood chemical items such as cholesterol, total protein, and triglycerides were not affected by FPS, lipid supplement or their interaction.

The blood serum insulin concentration was affected by lipid supplement ( $P=0.006$ ) and an interaction of

FPS×lipid supplement during the pre-weaning period ( $P=0.001$ ; Table 4) with lowest concentrations found for LP-SBO and highest concentrations found for LP-PLF. In the post-weaning period, serum insulin concentration was reduced by supplemental SBO compared to PLF ( $P=0.038$ ).

When further tested for the factor time, the concentrations of glucose total protein, triglycerides, cholesterol,

**Table 4** Least square means for blood metabolites and insulin concentration in dairy calves fed starters containing alfalfa hay with different particle sizes (small vs. long particles) with different lipid supplements (SBO vs. PLF, both 2.5% DM basis) ( $n=13$  calves per treatment)

Item	Treatments <sup>1</sup>				SEM	P-value <sup>2</sup>							
	SP		LP			FPS	Lipid	FPS×Lipid	T	FPS×T	Lipid×T	FPS×Lipid×T	
	SBO	PLF	SBO	PLF									
Glucose, mg/dL													
d 53	97.8 <sup>ab,B</sup>	94.2 <sup>b,B</sup>	89.9 <sup>B<sup>c</sup>,C</sup>	109.0 <sup>aA</sup>	5.09	0.26	0.029	0.003					
d 73	64.1 <sup>D</sup>	72.9 <sup>CD</sup>	69.2 <sup>CD</sup>	76.6 <sup>C</sup>	3.53	0.25	0.056	0.88					
Overall	81.0 <sup>ab</sup>	83.4 <sup>b</sup>	79.5 <sup>c</sup>	93.7 <sup>a</sup>	4.94	0.104	0.004	0.036	0.008	0.98	0.90	0.024	
BHB, mmol/L													
d 53	0.12 <sup>b,CD</sup>	0.11 <sup>b,CD</sup>	0.08 <sup>c,D</sup>	0.15 <sup>a,C</sup>	0.03	0.908	0.077	0.009					
d 73	0.25 <sup>B</sup>	0.33 <sup>A</sup>	0.31 <sup>AB</sup>	0.35 <sup>A</sup>	0.05	0.157	0.028	0.466					
Overall	0.19	0.22	0.19	0.25	0.04	0.188	0.017	0.495	0.003	0.22	0.26	0.052	
Total protein, g/dL													
d 53	7.22	7.16	7.12	7.28	0.057	0.970	0.818	0.606					
d 73	6.96	7.11	6.61	6.56	0.049	0.127	0.814	0.695					
Overall	7.08	7.14	6.86	6.92	0.061	0.195	0.736	0.985	0.036	0.18	0.92	0.51	
Triglyceride, mg/dL													
d 53	56.1	43.0	60.1	56.3	1.98	0.14	0.14	0.43					
d 73	33.5	26.3	28.0	30.2	0.79	0.83	0.51	0.21					
Overall	44.8	34.7	44.0	43.1	1.07	0.25	0.10	0.17	0.006	0.18	0.35	0.96	
Cholesterol, mg/dL													
d 53	103	107	109	116	6.5	0.29	0.43	0.86					
d 73	74.6	70.1	80.2	76.2	4.23	0.35	0.51	0.64					
Overall	72.3	105	78.1	112	5.98	0.15	0.91	0.87	0.001	0.93	0.30	0.94	
Blood urea nitrogen, mg/dl													
d 53	24.2	22.9	24.7	21.8	1.34	0.69	0.035	0.43					
d 73	16.5	18.2	17.8	16.8	1.02	0.94	0.88	0.27					
Overall	20.4	20.6	21.3	19.4	1.17	0.87	0.23	0.18	0.002	0.87	0.13	0.69	
Insulin, IU/L													
d 53	8.16 <sup>b,B</sup>	7.41 <sup>b,BC</sup>	6.63 <sup>c,C</sup>	9.65 <sup>a,AB</sup>	0.614	0.357	0.006	0.001					
d 73	9.96 <sup>AB</sup>	11.1 <sup>A</sup>	10.2 <sup>A</sup>	10.9 <sup>A</sup>	0.900	0.910	0.038	0.79					
Overall	9.0 <sup>b</sup>	9.26 <sup>b</sup>	8.36 <sup>c</sup>	10.3 <sup>a</sup>	0.893	0.554	0.012	0.001	0.007	0.55	0.88	0.009	

<sup>1</sup> Treatments: alfalfa hay provided as small particle size supplemented with soybean oil (SP-SBO); alfalfa hay provided as small particle size supplemented with palm fatty acids (SP-PLF); alfalfa hay provided as long particle size supplemented with soybean oil (LP-SBO); alfalfa hay provided as long particle size supplemented with palm fatty acids (LP-PLF)

<sup>2</sup> Statistical comparisons: Forage particle size (FPS) = small versus long particle sizes; Lipid = soybean oil versus palm fatty acids; FPS × Lipid = interaction between alfalfa hay particle sizes and supplemental lipid; T = time; FPS × T = forage particle size × time; Lipid × T = lipid source × time; FPS × Lipid × T = forage particle size × lipid source × time

<sup>a-c</sup> Different small letters indicate differences of a variable within one period (interaction FPS × Lipid;  $P \leq 0.05$ )

<sup>A-D</sup> Different capital letters indicate differences of a variable across periods (interaction FPS × Lipid × T;  $P \leq 0.05$ )



and blood urea nitrogen decreased ( $P=0.008$ ,  $0.036$ ,  $0.006$ ,  $0.001$ , and  $0.002$  respectively) whereas the concentrations of BHB and insulin increased ( $P=0.003$  and  $0.007$ , respectively) from pre-weaning to post-weaning. Three-way interactions of  $FPS \times lipid \times time$  existed for glucose, BHB and insulin ( $P=0.024$ ,  $0.052$  and  $0.009$ , respectively), which largely reflected the interaction of  $FPS \times lipid$  observed in the pre-weaning period.

Oxidative stress indicators GPx and SOD did not differ among experimental treatments; however, MDA was higher in calves receiving SBO compared to PLF during the pre- ( $P=0.024$ ) and post-weaning periods ( $P=0.005$ ) (Table 5). Malondialdehyde, GPx and SOD all increased from the pre-weaning to the post-weaning period ( $P=0.007$ ,  $0.034$  and  $0.052$ , respectively) without any two-or three-way interactions.

**Purine derivatives excretion and MPS**

Urine volume and urinary creatinine excretion were not affected by FPS, lipid supplement, and their interaction (Table 6). However, urinary excretion of allantoin, total PD and, hence, MPS were affected by lipid supplement during the pre-weaning ( $P=0.021$ ,  $P=0.036$ , and  $P=0.036$ , respectively) and post-weaning periods ( $P=0.004$ ,  $P=0.003$ , and  $P=0.003$ , respectively). Moreover, interactions between  $FPS \times lipid$  supplement showed that urinary allantoin, PD, and MPS were lowest for

LP-SBO and highest for LP-PLF during the pre-weaning ( $P=0.004$ ,  $P=0.008$  and  $P=0.008$ , respectively) and post-weaning periods ( $P=0.031$ ,  $P=0.042$ , and  $P=0.042$ , respectively). Creatinine, urine volume, allantoin, uric acid, total PD, and MPS all increased from the pre-weaning to the post-weaning period ( $P=0.004$ ,  $0.001$ ,  $0.009$ ,  $0.007$ ,  $0.005$ , respectively). Of note,  $lipid \times time$  interactions for allantoin, total PD and MPS ( $P=0.008$ ,  $0.001$ , and  $0.001$ , respectively) indicated that their values increased most prominently in the PLF groups from pre-weaning to post-weaning.

**Discussion**

The current research evaluated the effects of forage particle sizes (long particles vs. small particles) in combination with two lipid supplements (SBO vs. PLF) in starter diets of young dairy calves on starter intake, oxidative stress indicators, microbial protein synthesis, and growth performance. The experiment covered the pre-weaning and post-weaning periods. These periods greatly differ as weaned calves have a better-developed rumen and higher starter intake [8, 24] with the consequence that blood metabolites are more similar to a mature ruminant [30]. This was reflected by higher BHB and insulin concentrations and lower blood glucose concentrations compared to pre-weaned calves. Due to the higher feed intakes, calves post weaning also had accelerated growth

**Table 5** Least square means for oxidative stress indicators in whole blood of dairy calves fed starters containing alfalfa hay with different particle sizes (small vs. long particles) with different lipid supplements (SBO vs. PLF, both 2.5% DM basis) ( $n=13$  calves per treatment)

Item	Treatments <sup>1</sup>				SEM	P-value <sup>2</sup>						
	SP		LP			FPS	Lipid	FPS × Lipid	T	FPS × T	Lipid × T	FPS × Lipid × T
	SBO	PLF	SBO	PLF								
Glutathione peroxidase, IU/g Hb												
d 53	250	247	251	242	4.8	0.60	0.21	0.58				
d 73	297	289	299	293	5.1	0.47	0.14	0.88				
Overall	278	261	270	269	5.0	0.36	0.19	0.56	0.034	0.57	0.21	0.19
Superoxide dismutase, IU/g Hb												
d 53	2,267	2,351	2,240	2,215	83.8	0.34	0.73	0.52				
d 73	2,435	2,429	2,414	2,438	92.2	0.78	0.80	0.75				
Overall	2,390	2,389	2,374	2,364	91.6	0.54	0.70	0.34	0.052	0.60	0.43	0.27
Malondialdehyde, μmol/L												
d 53	2.41	1.81	2.16	1.89	0.093	0.11	0.024	0.39				
d 73	4.78	4.13	4.81	3.96	0.144	0.347	0.005	0.22				
Overall	3.89	2.90	3.07	2.17	0.132	0.236	0.062	0.78	0.007	0.79	0.093	0.45

<sup>1</sup> Treatments: alfalfa hay provided as small particle size supplemented with soybean oil (SP-SBO); alfalfa hay provided as small particle size supplemented with palm fatty acids (SP-PLF); alfalfa hay provided as long particle size supplemented with soybean oil (LP-SBO); alfalfa hay provided as long particle size supplemented with palm fatty acids (LP-PLF)

<sup>2</sup> Statistical comparisons: Forage particle size (FPS) = small versus long particle sizes; Lipid = soybean oil versus palm fatty acids; FPS × Lipid = interaction between alfalfa hay particle sizes and supplemental lipid; T = time; FPS × T = forage particle size × time; Lipid × T = lipid source × time; FPS × Lipid × T = forage particle size × lipid source × time

**Table 6** Least square means for urinary purine derivatives in dairy calves fed starters containing alfalfa hay with different particle sizes (small vs. long particles) with different lipid supplements (SBO vs. PLF, both 2.5% DM basis) ( $n = 13$  calves per treatment)

Item	Treatments <sup>1</sup>				SEM	P-value <sup>2</sup>							
	SP		LP			FPS	Lipid	FPS×Lipid	T	FPS×T	Lipid×T	FPS×Lipid×T	
	SBO	PLF	SBO	PLF									
Creatinine, mg/dL													
d 41–44	120	118	111	114	8.5	0.13	0.85	0.65					
d 66–69	138	133	139	143	12.7	0.26	0.94	0.26					
Overall	129	126	124	128	11.0	0.73	0.84	0.26	0.004	0.068	0.93	0.64	
Urine volume, L/d													
d 41–44	1.21	1.25	1.31	1.27	0.079	0.289	0.953	0.470					
d 66–69	1.71	1.78	1.71	1.64	0.146	0.304	0.891	0.229					
Overall	1.45	1.52	1.51	1.47	0.106	0.945	0.951	0.167	0.001	0.13	0.88	0.65	
Allantoin, mmol/d													
d 41–44	7.79 <sup>ab</sup>	7.56 <sup>ab</sup>	6.24 <sup>c</sup>	8.28 <sup>a</sup>	0.694	0.289	0.021	0.004					
d 66–69	13.1 <sup>b</sup>	15.8 <sup>ab</sup>	12.1 <sup>c</sup>	17.7 <sup>a</sup>	1.58	0.52	0.004	0.031					
Overall	10.4 <sup>b</sup>	11.7 <sup>ab</sup>	9.2 <sup>c</sup>	13.0 <sup>a</sup>	1.70	0.99	0.007	0.003	0.009	0.27	0.008	0.69	
Uric acid, mmol/d													
d 41–44	0.73	0.76	0.88	0.79	0.022	0.101	0.454	0.462					
d 66–69	1.16	1.19	1.25	1.18	0.082	0.622	0.608	0.385					
Overall	0.95	0.94	1.06	0.98	0.079	0.110	0.373	0.256	0.007	0.43	0.96	0.79	
Total PD, mmol/d													
d 41–44	8.53 <sup>ab</sup>	8.28 <sup>ab</sup>	7.11 <sup>c</sup>	9.08 <sup>a</sup>	0.894	0.436	0.036	0.008					
d 66–69	14.3 <sup>b</sup>	17.0 <sup>ab</sup>	13.4 <sup>c</sup>	18.9 <sup>a</sup>	1.73	0.51	0.003	0.042					
Overall	11.4 <sup>b</sup>	12.6 <sup>ab</sup>	10.2 <sup>c</sup>	13.9 <sup>a</sup>	1.59	0.86	0.014	0.028	0.005	0.33	0.001	0.72	
MPS, g/d <sup>3</sup>													
d 41–44	45.6 <sup>ab</sup>	44.3 <sup>ab</sup>	38.0 <sup>c</sup>	48.5 <sup>a</sup>	2.64	0.43	0.036	0.008					
d 66–69	76.8 <sup>b</sup>	91.2 <sup>ab</sup>	70.4 <sup>c</sup>	101 <sup>a</sup>	6.78	0.51	0.003	0.042					
Overall	61.2 <sup>b</sup>	67.8 <sup>ab</sup>	54.9 <sup>c</sup>	74.8 <sup>a</sup>	5.90	0.86	0.010	0.023	0.005	0.33	0.001	0.72	

<sup>1</sup> Treatments: alfalfa hay provided as small particle size supplemented with soybean oil (SP-SBO); alfalfa hay provided as small particle size supplemented with palm fatty acids (SP-PLF); alfalfa hay provided as long particle size supplemented with soybean oil (LP-SBO); alfalfa hay provided as long particle size supplemented with palm fatty acids (LP-PLF)

<sup>2</sup> Statistical comparisons: Forage particle size (FPS) = small versus long particle sizes; Lipid = soybean oil versus palm fatty acids; FPS × Lipid = interaction between alfalfa hay particle sizes and supplemental lipid; T = time; FPS × T = forage particle size × time; Lipid × T = lipid source × time; FPS × Lipid × T = forage particle size × lipid source × time

<sup>3</sup> Microbial protein synthesis (MPS) was estimated from urinary purine derivatives (PD) excretion based on Chen and Gomes [33] as  $MPS (g/d) = 70 \times PD (mmol/d) / (0.85 \times 0.116 \times 0.83 \times 1,000) \times 6.25$

<sup>a–c</sup> Different small letters indicate differences of a variable within one period (interaction FPS × Lipid;  $P \leq 0.05$ )

compared to pre-weaned calves. Importantly, the joined consideration of these periods provided a coherent picture of the interaction effects of lipid source and FPS. Francisco et al. [34] had evaluated such effects previously in lambs; however, limited work is published on this interaction in dairy calves.

We chose a forage inclusion level (on a DM basis) of 10% in accordance with Aragona et al. [35] who suggested that 5% forage incorporation constitutes the minimum requirement to support optimum development of ruminal fermentation. With respect to FPS, previous studies reported beneficial effects of increasing the particle size of alfalfa hay on calf performance

and nonnutritive oral behaviors [36] as well as starter intake and weaning weight (DM basis) [8]. Omidi-Mirzaei et al. [37] evaluated wheat straw particle sizes when calves were fed texturized starters and also reported improved feeding behavior and ruminal fermentation when the content of fine particles was reduced. The novel finding in the current study is that lipid supplement becomes decisive for the effect of FPS when diets are lipid-supplemented. A positive effect of long FPS could only be observed when a rumen-inert source of fatty acids (PLF) was used for dietary fat supplementation. For example, animals in the LP-PLF group had higher post-weaning ADG, final BW, and higher serum

glucose, BHB and insulin concentrations compared to animals in the SP-PLF group.

In contrast to PLF, starter intake during the pre-weaning period was greatly reduced by SBO. Moreover, an interaction between FPS×lipid supplement indicated that a long FPS, which was rather beneficial with PLF, enforced the negative effects of SBO on pre-weaning starter intake, resulting in a dramatic reduction in starter feed intake in group LP-SBO compared to LP-PLF by 270 g/d. When acknowledging that this period is critical for rumen development in young calves, it is understandable that performance parameter like ADG and withers height were reduced later in the post-weaning period in which animals of LP-SBO additionally showed the lowest value for feed conversion.

Soybean oil is rich in unsaturated FA in contrast to PLF that contains high level of saturated FA [12, 23, 38]. Unsaturated FAs were previously shown to reduce digestibility of NDF in dairy calves, which was exacerbated upon forage inclusion in starter diets [11]. This partly conforms with the results of the present study where a lower digestibility was indicated for OM and NDF selectively in calves receiving the SBO diet; however, only when FPS was long. This lower digestibility of nutrients is one likely reason that explains concurrent suppression of feed intake. Apart from calves [23], a feed intake depression due to unsaturated FAs is well documented in mature ruminants [39, 40]. Toxic compounds have been identified as a cause by which certain supplemental lipids can reduce NDF digestibility in ruminants [41]. It is perceivable that this phenomenon is aggravated in young dairy calves that have not fully established fiber-digesting bacteria in their rumen. The role of protozoa in fiber digestion can also be negatively affected by lipid supplementation in ruminants [42]. Collectively, the results support that LP may be more favorable than SP from the ruminal fermentation perspectives [8, 36], even if moderate amounts of saturated fatty acids are included in the diet. However, it is highly recommendable to reduce FPS when calves shall receive starters supplemented with oil rich in unsaturated fatty acids to avoid strong depressing effects on fiber digestion and feed intake. It may be speculated that unsaturated fatty acids can have stronger coating effect on LP vs. SP due to the smaller surface area of LP, leading to stronger impairment of microbial attachment on LP vs. SP.

According to the lowest starter intake in LP-SBO, the lowest ADG (658 g/d) during the post-weaning period was found in calves receiving LP-SBO, coinciding with lowest withers girth at weaning (90.7 cm) and final measurement (96.1 cm). These results show that feeding starters containing LP alfalfa hay with SBO supplementation provide insufficient nutrients for acquiring

gain in young dairy calves. However, calves receiving alfalfa hay in the form of LP size had greater barrel size which may be related to greater gut fill rather than being an indicator of improved growth rate. Previous studies also stated that feeding forage to young dairy calves may confound results on growth performance due to increased fill of the gastrointestinal tract rather than a true effect on growth [8, 43].

Our result clarified that feeding SBO along with LP provided the lowest digestibility of OM (66.8%). We did not measure ruminal fermentation in the current study, but because ruminal fermentation and short fatty acid production is strongly dependent on OM fermentation in the rumen [44], a lower ruminal fermentation rate can be postulated for the LP-SBO diet. This postulate takes into account both the lower starter (i.e., OM) intake and the lower OM digestibility. In addition, looser feces in calves of the two SBO-supplemented groups pre-weaning, with highest fecal scores in the group SP-SBO, indicated that lower digestibility of OM and NDF is additionally related to higher passage rate, which would coincide with reduced microbial activity [23].

Glucose and BHB concentrations in the blood of calves fed the LP-SBO diet were reduced, which fits the lower starter intake detected in this group. As a result of lower starter intake and lower digestibility, fewer nutrients can be provided for absorption in the gastrointestinal tract and, hence, glucose and BHB were reduced as energy level indicators in the LP-SBO group. Higher concentrations of BUN in both groups supplemented with SBO compared to PLF indicate that ammonia nitrogen concentration was higher in the rumen of calves supplemented with the unsaturated FA source, which would be in line with previous studies [44]. This indicates lower nitrogen utilization efficiency in SBO vs. PLF diets due to lower microbial activity in the rumen.

In agreement with the reduced serum glucose and BHB concentrations, the concentration of insulin was also reduced (6.63 IU/L) when calves were fed LP-SBO. Because insulin is a function of glucose concentration, the lower insulin concentration logically followed the lower glucose concentration. Lower insulin concentration, in turn, may contribute to lower growth in young ruminants because of its role in glucose and amino acid metabolism that is critical in the early stage of growth [29, 45]. By contrast, the highest pre-weaning and overall concentrations of glucose, BHB and insulin were observed in group LP-PLF. This supports the notion that it is not the inclusion of LP but clearly the combination of LP with SBO that decreases metabolic and endocrine growth signals. In agreement with this postulate, a recent study by Takemura et al. [2] indicated that forage

inclusion in starter diets of dairy calves increased growth hormone concentration in their blood.

Further indications for the mechanisms behind impaired performance of LP-SBO calves were obtained from the estimation of MPS. Calves in the group LP-SBO had lowest urinary allantoin and total urinary PD excretion during pre-weaning and post-weaning, indicating lowest MPS. The MPS estimated based on urinary PD can be used as an indicator for microbial ruminal development in young calves [30]. The reduction of allantoin and urinary PD excretion detected in calves fed LP-SBO thus suggests lower microbial activity as a plausible cause for decreased OM and NDF digestibility. Of note, starter intake and, as such, the amount of OM provided to microbes, was also reduced in the pre-weaning period in group LP-SBO, thus withdrawing OM from ruminal microbes as a key factor to support microbial protein synthesis [46].

A last aim of the present study was to assess the interaction effect of FPS $\times$ lipid on oxidative status. The concentrations of MDA were lower in the blood of calves that received PLF compared to SBO diets. MDA has been considered as an important indicator for oxidative damage in pre-weaned dairy calves [47]. Calving leads to oxidative stress, which can increase the formation of reactive oxygen species and overwhelm the antioxidant systems of neonate calves [47, 48]. Our results suggest that regardless of forage particle size, supplementing starters with unsaturated FA sources can aggravate oxidative stress in young calves, especially during the pre-weaning period. Although limited documents are available on the effect of supplemental lipid on oxidative stress indicators in dairy calves [48], Tsai et al. [49] indicated that SBO rich in linoleic acid has some pro-inflammatory effect in young ruminants that can modulate intake and performance in pre-weaning calves. The ruminal metabolism of FA should also be considered with caution when lipid supplementation is provided to dairy cows [50, 51], calves [52] or lambs [14, 15]. Most of the negative responses to supplemental unsaturated FA had been attributed to its negative influence on ruminal fermentation and microbial activity in calves under 2 months of age [13, 46]; however, it seems that additional effects on energy metabolism and its hormonal regulation, as well as on oxidative stress and possibly inflammation, should receive additional attention in future studies.

## Conclusions

A strong interaction of dietary forage particle size $\times$ lipid supplement was found in young dairy calves. With a rumen-inert fatty acid source (palm fatty acids), we observed a measurable increase of average daily gain, serum glucose, beta-hydroxy butyrate and

insulin levels pre-weaning, as well as increased neutral detergent fiber digestibility and final body weight, when increasing particle size (1.26 mm to 4.97 mm) in a starter diet containing 10% alfalfa hay (dry matter basis). This is compatible with the effect of forage particle size observed using forage-supplemented starters without lipid supplementation in previous studies. By sharp contrast, addition of soybean oil to the starter diet had a generally depressing action on several of the above mentioned readouts that became obvious or was aggravated when increasing forage particle size. Therefore, supplementation with 25 g/kg palm fatty acids can be recommended to increase the energy density of forage-containing starter diets for dairy calves and preference should be given to a long forage particle size. On the contrary, negative performance effects of soybean oil as lipid supplement are especially evident with a long forage particle size. If soybean oil is to be used in starter diets for young dairy calves, the particle size should be short to avoid or ameliorate depressions of intake, digestibility, ruminal microbial activity, and growth performance.

## Abbreviations

ADG	Average daily gain
BHB	Beta-hydroxy butyrate
BW	Body weight
CP	Crude protein
DM	Dry matter
FA	Fatty acid
FE	Feed efficiency
FPS	Forage particle size
GPx	Glutathione peroxidase
LP	Long particles
MDA	Malondialdehyde
MPS	Microbial protein synthesis
NASEM	National Academies of Science, Engineering, and Medicine
NDF	Neutral detergent fiber
NRC	National research council
OM	Organic matter
PD	Purine derivatives
PLF	Palm fatty acids
SBO	Soybean oil
SP	Small particles
SOD	Superoxide dismutase

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## Authors' contributions

PP Data curation, funding acquisition, laboratory analysis; HMA and MKB Conceptualization, methodology, supervision, writing—original draft, writing—review and editing, project administration, funding acquisition; MP Resource, data validation and evaluation; MV Methodology, formal analysis, data validation and evaluation; JRA Formal analysis, writing—review and editing. All authors read and approved the final manuscript.

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#### Availability of data and materials

The datasets used and/or analyzed in the current study are available from the authors on reasonable request.

#### Declarations

#### Ethics approval and consent to participate

The experimental design, management protocols, and all procedures were approved by the Animal Care Committee at the University of Zanjan, Iran (ID 1353).

#### Consent for publication

Not applicable.

#### Competing interests

The authors declare no real or perceived conflicts of interest.

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