

The effects of supplementing yeast fermentation products on gut permeability, hormone concentration, and growth in newborn dairy calves¹

Lucía Pisoni, Alejandro E. Relling^{2,*}

Department of Animal Sciences, The Ohio State University, Ohio Agricultural Research and Development Center, Wooster, OH 44691

ABSTRACT: The objectives of this study were to evaluate the effect of the use of yeast fermentation products (YFP) on growth, hormone concentration, and gut permeability in dairy calves. One hundred and twenty heifers were randomly assigned to one of three treatments: control group with no YFP supplementation (C), *Saccharomyces cerevisiae* fermentation products (SCFP) supplementation (1 g/head/d of SmartCare [Diamond V] in the milk and 0.7% on dry matter basis of NutriTek [Diamond V] on the starter feed), or *Aspergillus oryzae* fermentation extracts (AOFE) supplementation (3 g/head/d of LXtract1224 [Biozyme Inc.] in the milk). All calves received 6 L/d of pasteurized milk and had ad libitum access to water and dry feed along the study. Body weight (BW) was recorded at birth and on days 14, 30, and 45 and at weaning. Dry feed (starter) offered was measured daily and refusals twice a week to obtain starter intake (SI). Diarrhea events were recorded daily and fecal scores were classified by using a four-point scale. Blood was sampled on days 7 and 14 for plasma glucose, nonesterified fatty acids (NEFA), insulin, and IL-1 β concentrations. Lactulose and D-mannitol were included in the morning feeding of day 14 and blood samples were taken an

hour after feeding for assessment of intestinal permeability. On day 14, blood samples were taken for plasma glucagon-like peptide 2 (GLP-2) concentration. On day 30, fecal samples were collected for measurements of *Salmonella* and *Escherichia coli* concentration on feces. No treatment differences ($P \geq 0.13$) were found for BW or SI. There was a time by treatment difference ($P = 0.01$) in average daily gain (ADG) on day 45 where C animals had a greater ADG when compared with SCFP and AOFE. Diarrhea incidence did not change between treatments ($P = 0.97$) and *Salmonella* and *E. coli* were not found in feces. There were no differences ($P > 0.60$) between treatments for plasma GLP-2, glucose, insulin, lactulose, nor D-mannitol concentrations. There was a time by treatment tendency ($P = 0.06$) for NEFA concentration which tended to be greater on day 7 for C and AOFE when compared with day 14. Plasma IL-1 β concentration showed a treatment tendency which tended ($P = 0.06$) to be greater for SCFP when compared with C. Under the current conditions, supplementation with YFP did not improve performance parameters. Plasma GLP-2 concentration, intestinal permeability, and plasma metabolites did not differ after yeast fermentation products supplementation.

Key words: dairy calf, gut peptides, leaky gut, prebiotics

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²Corresponding author: relling.1@osu.edu

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INTRODUCTION

Raising heifer calves as replacement animals is one of the most important activities on dairy farms. These animals will represent the source of new genetics and the basis for the development of the herd. The occurrence of infectious diseases is well known to take place in this period generating important losses. Diarrhea is the main cause of morbidity and mortality in calves during their early development (U.S. Department of Agriculture, 2007), becoming the major cause of economic losses to calf producers (Cho and Yoon, 2014). Diarrhea is typically treated with antibiotics. However, the use of antibiotics to treat diarrhea, as well as their use as growth promoters, has been associated with the generation of antibiotic resistance and the development of resistant strains of bacteria (Fuller, 1989). Their use has also generated consumers' concern in the last years due to the potential reservoir of antibiotic-resistant bacteria in food products (Smith et al., 2002). In this scenario, the search for an alternative to antibiotics becomes crucial. Prebiotics, and more specifically yeast fermentation products (YFP), have been shown to improve performance in poultry (Hajati and Rezaei, 2010) and to increase indices of immunity and intestinal health in swine production (van der Peet-Schwering et al., 2007). Prebiotics have been described as nondigestible food ingredients that beneficially stimulate growth and activity of bacteria in the gut (Gibson and Roberfroid, 1995). These compounds could be considered as an alternative to improve dairy calves' health and performance. Raising healthier calves would also decrease diarrhea incidence and, as a result, the use of antibiotics to treat it. Even though these products have been widely implemented in several species and are broadly commercialized, research has shown inconsistencies in their physiological responses through the literature (Alugongo et al., 2017). These inconsistencies might be related to their unknown mechanism of action and to specific variations found within trials, such as the strain of yeast used, doses, immune status of the

animals, management, feed quality, and number of animals enrolled, among others. Some studies in calves have demonstrated a positive association between the use of YFP and performance and health parameters, such as dry matter intake (DMI), average daily gain (ADG; Lesmeister et al., 2004), rumen papillae development (Xiao et al., 2016), decreased diarrhea incidence (Brewer et al., 2014), and immune responses (Harris et al., 2017). However, YFP beneficial effects have been mostly associated with pathogen-challenged models (Alugongo et al., 2017) and there is no much information in relation to nonpathogen-challenged trials on calves. On the other hand, supplementation with a prebiotic similar to YFP stimulated the secretion of the gut hormone glucagon-like peptide 2 (GLP-2) by inducing changes in gut microbiota in a mice model (Cani et al., 2009). This hormone is positively associated with intestinal permeability regulation. GLP-2 has been probed to stimulate intestinal mucosal growth and increase mesenteric blood flow (Taylor-Edwards et al., 2011). It was also shown (Walker et al., 2015) that GLP-2 improves gut integrity by stimulating the expression of tight junction proteins in the intestine of Holstein calves.

Thus, the hypothesis of this study is that the use of YFP as a supplement to dairy calves' diet not only improves performance but also increases plasma total GLP-2. The increased concentration of this hormone is associated with a decrease in gut permeability. By decreasing gut permeability, the incidence of diarrhea on dairy calves in the pre-weaned stage will decrease. The objectives of the current study were to evaluate the effect of the use of YFP on growth, hormone concentration, and gut permeability in dairy calves as the association of factors that might increase gut permeability.

MATERIALS AND METHODS

Treatment, Animals, and Feeding

This research study was conducted at Ayers Dairy Farm, located in Perryville, OH (IACUC

#2017A00000015). For this experiment, 120 newborn dairy heifers (initial body weight [BW] = 40.5 kg average) were enrolled in a complete randomized block design at birth and used until weaning at day 56 ± 3 . These animals were blocked by birth date and BW and assigned to one of three different treatments: control treatment (C, $n = 40$), *Saccharomyces cerevisiae* fermentation products (SCFP) supplementation treatment ($n = 40$), and *Aspergillus oryzae* fermentation extracts (AOFE) supplementation treatment ($n = 40$). Control group did not receive YFP supplementation; SCFP group was supplemented with 1 g/head/d of SmartCare (Diamond V) in the milk and 0.7% on dry matter (DM) basis of NutriTek (Diamond V) on the starter feed; AOFE group was supplemented with 3 g/head/d of LXtract1224 (Biozyme Inc.) in the milk. Doses were based on the vendor's recommendations. Animals were raised in individual hutches equipped with two buckets per hutch, one for the milk and one for the dry feed (starter). Milk was delivered twice a day, from 0600 to 0800 hours and from 1500 to 1630 hours. Treated groups received their treatments in the morning feeding from birth until weaning. All calves were bottle fed 6 L of colostrum during the first three feedings during the first 36 h following birth. The first colostrum intake was immediately after birth. Animals had an adequate colostrum protocol where colostrum quality was ensured and supplemented with artificial colostrum when needed. From the fourth feeding until weaning, calves received 3 L of pasteurized milk twice a day in the buckets. During milk delivery and consumption, calves were individually controlled to ensure that they consumed all the milk. If a calf did not drink all the milk, it was helped and trained by the farm personnel to ensure complete consumption of the milk in the bucket. Whenever an animal did not consume the entire milk allocation, this was recorded and that animal stayed under observation in case of eventual occurrence of disease. Animals had ad libitum access to water from day 1 and starter feed from day 3. Starter feed was based on a commercial diet containing 24.6% of crude protein, 6.9% Acid detergent fiber (ADF), 9.87% Neutral detergent fiber (NDF), 2.3% fat, 40.9% starch, 0.8% calcium, and 0.5% phosphorus. Grass hay was provided ad libitum starting on day 30. Hay was provided in order to stimulate rumen growth; however, its intake was not recorded. Calves were mostly experimenting on how to consume this pasture and this caused that most of the hay ended up being wasted rather than consumed. In addition, calves were able to take hay from neighboring hutches.

On day 56 ± 3 , animals were weaned and finished the trial. Calves' records of treatments, diarrheas, and colostrum intake, among others, were daily recorded.

Sample Collection and Analysis

Between 24 and 48 h after birth, a blood sample was taken from the jugular vein from all calves to determine basal concentration of immunoglobulin G (Ig G) on serum. Blood samples were collected on vacutainers containing silica and a polymer gel to facilitate serum separation (BD Vacutainer SST). After arrival to the laboratory, blood samples were centrifuged for 20 min at $2000 \times g$ and 4°C to separate the serum. Serum total protein (STP) concentration was measured by using a clinical refractometer (Jorgensen refractometer, J-351, Loveland, CO). The use of a clinical refractometer facilitated early detection of failures on the passive transfer of immunity and the selection of only those animals with a correct immunity at the beginning of the trial. The remaining serum was aliquoted in individual polypropylene tubes and stored at -20°C until analysis. Plasma concentration of IgG was later determined by radial immunodiffusion test (Triple J Farms, Bellingham, WA). Samples were run on duplicates. Calves' BW was measured at birth and on days 14, 30, and 45 and at weaning (on day 56 ± 3).

Since day 3, calves were supplemented starter feed. The starter was weighed in 500-g (DM basis) bags and separated by color tags according to the corresponding treatment group. Animals under SCFP treatment received 3.5 g of YFP mixed with the starter. For the SCFP group, after the addition of 3.5 g of YFP, the plastic bag was closed and shaken to ensure mixing. Whenever the amount of grain in the bucket was less than 2.5 cm, a new 500-g bag was added, a visual examination of the bucket was conducted twice a day. DM offered was measured daily and refusals twice a week to obtain an average starter intake (SI). Refusals were collected, sampled in duplicates and consequently dried in a forced air oven at 100°C for 24 h. After 24 h, refusals' DM was calculated. Based on those results, daily SI was calculated for each calf.

Fecal scores were measured daily by following a four-point scale as follows: 1 = normal; 2 = soft to loose; 3 = loose to watery; 4 = watery, mucous, and bloody. Following the farm standard operation protocol, animals presenting a fecal score ≥ 3 were considered to be diarrheic and treated with 3 mL of ceftiofur hydrochloride for 5 d. Diarrhea

records were taken for all animals during the entire trial. Records included the score and the times an animal experienced a diarrhea event. This information was later used to calculate diarrhea incidence.

On day 14, lactulose and D-mannitol sugars were added into the buckets and mixed with the morning milk in a concentration of 21 g/31.5 mL and 133.3 g/31.5 mL, respectively. The concentrations of these sugars and the day of sampling were based on a previous study (Araujo et al., 2015). In Araujo et al. (2015), sugars were sampled on days 0, 7, 14, and 21, and both days 7 and 14 showed increments of lactulose in plasma in calves with diarrhea compared with calves with no incidence of diarrhea. The reason why we chose day 14 over day 7 is because we wanted to provide enough time for the YFP to have an actual effect on the gut before measuring gut permeability. Blood samples were obtained 1 h after feeding the markers to evaluate gut permeability based on sugars' concentrations. Sugars were prepared in a liquid state in order to facilitate mixing with the milk. Sugars were delivered following treatments and a multi-dose syringe was used to calculate doses and to distribute the sugars in each bucket. After delivering the sugars, mixing with the milk was insured and controlled for each bucket. Plasma extraction of lactulose and D-mannitol was performed using a liquid-liquid extraction. For this procedure ^{13}C glucose and ^{13}C fructose were added as internal standards. Samples were extracted as follows: 100 μL of the plasma samples were mixed with 10 μL of internal or external standards, separately. Following, 300 μL of methanol/chloroform/hexanes (2:0.5:0.5 by volume), 100 μL of 100% chloroform and 100 μL of double-distilled water were added to the mixture and vortexed thoroughly. The solution was next centrifuged at $17,000 \times g$ at 23°C for 15 min. The supernatant was then collected and transferred to a new microcentrifuge tube containing 250 μL of distilled water and centrifuged again at $17,000 \times g$ at 23°C for 5 min. Finally, 350 μL of the sample were transferred to an Amicon 3kDa filtering device and centrifuged at $14,200 \times g$ for 45 min at room temperature before liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. Lactulose and D-mannitol measurements were performed by LC-MS/MS as previously described (Armirotti et al., 2014).

On days 7 and 14, blood samples were taken for measurements of plasma concentration of glucose, nonesterified fatty acids (NEFA),

interleukine- 1β (IL- 1β), and insulin. Blood was obtained from the jugular vein and immediately transferred to polypropylene tubes containing Ethylenediaminetetraacetic acid (EDTA) and benzamidine hydrochloride 99% used as a protease inhibitor. Samples were placed on ice until arrival at the laboratory where they were centrifuged for 20 min at $2000 \times g$ and 4°C and finally stored at -20°C until analysis. All samples were run in duplicates. Plasma concentration of glucose was measured using a colorimetric assay (Trinder #1070, Stanbio Laboratory, Boerne, TX). The intra-assay coefficient of variation was 3.09%. Plasma NEFA concentrations were measured using microtiter plates and a plate reader in an enzyme-based assay (Wako Chemicals USA, Inc., Richmond, VA) as previously described (Johnson and Peters, 1993). The intra-assay coefficient of variation was 3.14%. Plasma concentration of IL- 1β was performed using a commercial kit (bovine IL- 1β , ESS0027, Invitrogen, Thermo Fisher Scientific, Vienna, Austria) and following manufacturer instructions. The intra-assay coefficient of variation was 7.53%. Plasma concentration of insulin was measured by using a commercial kit (Porcine insulin, RIA PI-12K, Millipore, Burlington, MA). A validation was conducted based on parallel displacement of insulin binding by incremental addition of bovine plasma and compared with an insulin standard curve and by recovery of swine insulin on bovine plasma ($97 \pm 5\%$ recovery). The intra-assay coefficient of variation was 6.8%.

Plasma concentration of GLP-2 was measured from plasma samples collected on day 14. Blood was obtained as described for previous metabolites with the only exception that GLP-2 samples were stored at -80°C until analysis. Plasma total GLP-2 concentration was measured using a commercial kit (FEK-028-14; Phoenix Pharmaceuticals, Phoenix Europe GmbH, Karlsruhe, Germany). A validation was conducted based on parallel displacement of GLP-2 binding by incremental addition of bovine plasma and compared with a GLP-2 standard curve and by recovery of human GLP-2 on bovine plasma ($89 \pm 18\%$ recovery). The intra-assay and inter-assay coefficients of variation were 2.88% and 17.07%, respectively.

On day 30 of the study, a fecal sample was collected from 80 animals (C = 26, SCFP = 25, and AOFE = 29) for measurements of *Salmonella* and *Escherichia coli* presence on feces. Each bacterium was grown on a specific selective media. First, each

sample was subjected to a 5-fold dilution with saline solution to facilitate pipetting. Samples were then diluted in a 96-well plate with saline solution. Then, the specific media were prepared for each bacteria. Agar MacConkey was used for *E. coli* proliferation, and Agar XLT was used for *Salmonella* culturing. An enrichment media of tetrathionate broth was later prepared to stimulate *Salmonella* growth. All readings were made after 24 h of incubation.

Statistical Analysis

Along the experiment, one calf from the SCFP group (11 d of age) and two calves from the C group (11 and 10 d of age, respectively) died from unknown causes. Only serum samples from day 1 and blood samples from day 7 of the study were used from these animals. The remaining 117 newborn calves finished the trial. Data were analyzed as a randomized complete block design with repeated measurements for those continuous variables with multiple sampling over time using the MIXED procedure of SAS (9.4, Institute, Cary, NC). The model included the random effect of calves within block and the fixed effect of treatment, time, and their interactions. The compound symmetry structure and the Kenward–Roger degrees of freedom were used based on the lower Akaike's information criterion value. Because of expected differences in variance due to treatments, the statement "GROUP = treatment" was used to separate SEM and left in the model if the Akaike's information criterion value was lower. The option "SLICE" was used to separate means at each specific sampling time when time by treatment interactions were significant. The variables with a single sampling were run with a similar model but without time and its interaction. The GLIMMIX procedure with binomial distribution was used to evaluate the incidence of diarrhea. The model included the random effect of calves within block and the fixed effect of treatment. Mean comparisons were conducted using the "PDIF" statement of SAS whenever there was a treatment effect or tendency. Multiple linear regression was used to evaluate the effect of plasma GLP-2, IL-1 β , and Ig G concentrations, the incidence of diarrhea at the sampling time, and SI at day 14 on the sugars' ratio (lactulose:D-mannitol). Also, a multiple linear regression was used to evaluate the effect of SI (average at day 14), sugars' ratio, and plasma insulin concentration on ADG (average at day 14). The model included the

fixed effect of treatment. Significant results were settled at $P \leq 0.05$ and tendencies at $P \geq 0.05$ and $P \leq 0.10$.

RESULTS AND DISCUSSION

Plasma Ig G Concentration

Serum concentration of Ig G and serum total protein (STP) are two commonly used techniques for assessment of passive transfer of immunity. In healthy calves, an STP concentration of 5.2 g/dL or greater is associated with a correct passive transfer of immunity (Tyler et al., 1996), whereas serum concentrations of Ig G greater than 1,000 mg/dL are considered to reflect an adequate passive transfer of immunity (McGuirk and Collins, 2004). Results from this study showed that there were no differences between treatments ($P > 0.1$; Table 1) in relation to plasma Ig G concentration. This means that none of the enrolled calves presented the failure of passive transfer of immunity at the beginning of the trial. Thus, further results found in this study were not influenced by these animals' immune status.

Performance

No differences between treatments were found in BW or SI ($P \geq 0.13$; Figures 1 and 2, respectively). As expected, SI and BW increased from birth till weaning, stimulated by the increase in the intake of starter feed. These results are in accordance with previous research (Beharka et al., 1991; Yohe et al., 2015). Yohe et al. (2015) found no differences in DMI, BW, and ADG on 52 bull calves fed 2 g/d of a supplement based on AOFE. Similarly, 72 Holstein heifer calves fed low, moderate, or high (0.92,

Table 1. Assessment of passive transfer of immunity by measuring serum Ig G and serum total protein concentration in newborn dairy calves between 24 and 48 h after birth^a

Item	Treatments ^b				P-value
	C	AOFE	SCFP	SEM	Trt ^c
Serum Ig G (mg/dL)	3,700	3,791	3,737	182.2	0.93
Serum total protein (g/dL)	6.1	6.1	6.2	0.11	0.82

^aData are presented as least square means \pm SEM.

^bC = not supplemented; AOFE = 3 g/head/d of LXtract 1224 (Biozyme Inc.) in the milk; SCFP = 1 g/head/d of SmartCare (Diamond V) in the milk and 0.7% on DM basis of NutriTek (Diamond V) on the starter feed.

^cTreatment.

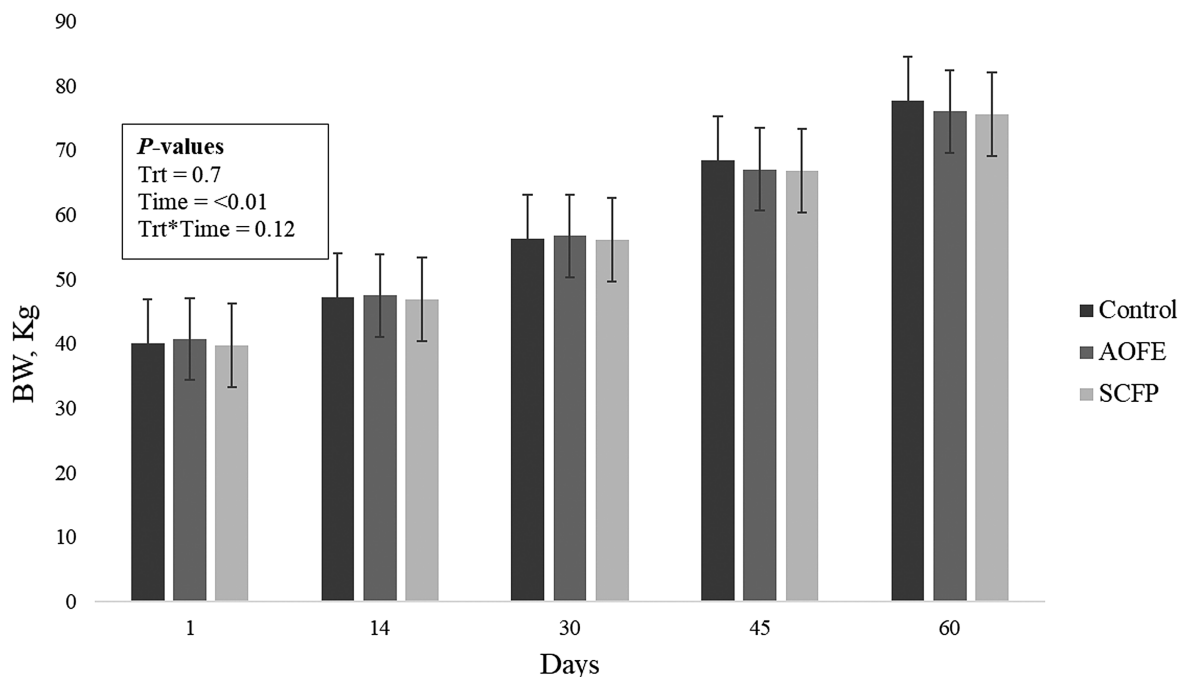


Figure 1. Effect of yeast fermentation products' supplementation on BW of newborn dairy calves. Control = not supplemented; AOFE = 3 g/head/d of LXtract1224 (Biozyme Inc.) in the milk; SCFP = 1 g/head/d of SmartCare (Diamond V) in the milk and 0.7% on DM basis of NutriTek (Diamond V) on the starter feed. Data are presented as least square means \pm SEM.

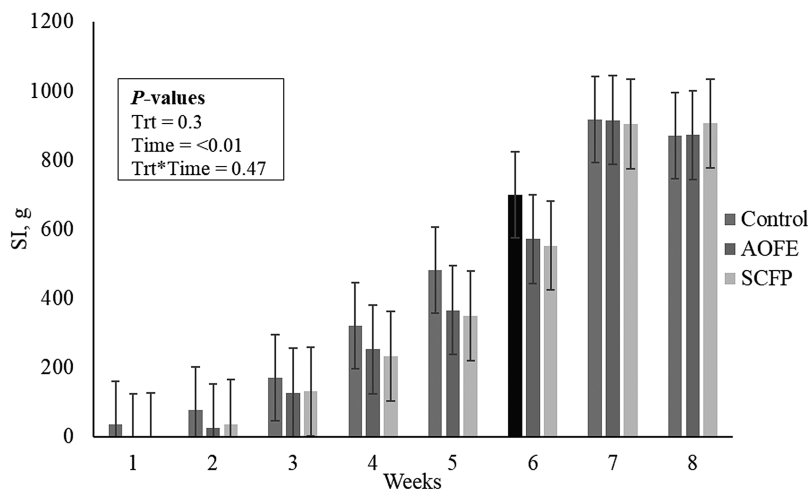


Figure 2. Effect of yeast fermentation products' supplementation on SI of newborn dairy calves. Control = not supplemented; AOFE = 3 g/head/d of LXtract1224 (Biozyme Inc.) in the milk; SCFP = 1 g/head/d of SmartCare (Diamond V) in the milk and 0.7% on DM basis of NutriTek (Diamond V) on the starter feed. Data are presented as least square means \pm SEM.

1.83, and 5.5 g/kg, respectively) concentrations of AOFE did not show any treatment differences in DMI, weekly weight gain, and feed conversion ratio (Beharka et al., 1991). Results from the present study are in accordance with previous research conducted on 512 Holstein calves fed *S. cerevisiae* fermentation extracts for a period of 70 d where no differences between treatments were found in BW (Magalhães et al., 2008). Increments in BW during the first months of life tend to be limited. During the preweaned stage, milk is the basis of the feed; however, because milk is fed at restricted amounts, calves tend to maintain or gain little BW (Davis

and Drackley, 1998). In addition, consumption of starter feed varies considerably between animals. In our study, some calves showed a faster acceptance to the dry feed, while others took longer to familiarize and start eating it. There was a time by treatment difference in ADG ($P = 0.01$; Figure 3). During the study, ADG was similar for all the periods but one. On day 45 of the study, C-treated animals had an ADG of 0.84 kg/day, while SCFP and AOFE had an ADG of 0.68 and 0.67 kg/day, respectively. However, ADG at the end of the trial (56 ± 3 d) was similar among treatments. On the contrary, Lesmeister et al. (2004) found differences

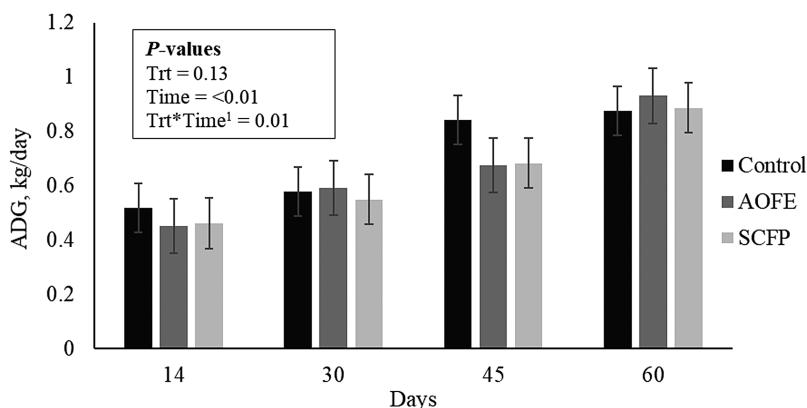


Figure 3. Effect of yeast fermentation products' supplementation on ADG of newborn dairy calves. Control = not supplemented; AOFE= 3 g/head/d of LXtract1224 (Biozyme Inc.) in the milk; SCFP = 1 g/head/d of SmartCare (Diamond V) in the milk and 0.7% on DM basis of NutriTek (Diamond V) on the starter feed. Data are presented as least square means \pm SEM. ¹Slice option of SAS were used for mean separation for the time by treatment interaction, reporting difference in ADG on day 45 ($P < 0.01$).

in DMI, ADG, and BW in calves treated with SCFP. The doses used by Lesmeister et al. (2004) were 1% and 2%, calculated as a percentage of the DM. In our study, 1 g/head/d of SmartCare (Diamond V) in the milk and 0.7% on DM basis of NutriTek (Diamond V) on the starter feed were supplemented. Our dose changed as a percentage of the total SI of SCFP, which was approximately 11% of the starter intake in week 1 and 0.81% in week 7. The reason for this big difference is the low intake of starter in week 1. However, animals were also receiving 1 g of SCFP in the milk, whereas the dose used by Lesmeister et al. (2004) was strictly dependant on the YFP included in the starter feed since no supplementation was given with the milk. Despite both products being SCFP, it is possible that differences in dose or feeding management might have affected the responses. Another difference is related to weaning time. In Lesmeister et al. (2004), calves were abruptly weaned at day 35 ± 1 of age but stayed in the study until day 42 ± 1 of age; and it was at this moment when differences on DMI were observed. In our study, animals were weaned at day 56 ± 3 of age. We did find a time by treatment difference on ADG, where ADG was similar in all periods but different on day 45; however, C-treated animals were the ones exhibiting improvements in ADG and not YFP-treated groups. In addition, these differences were no longer observable by weaning time. It was assumed that YFP will cause increments in performance by stimulating gut microbiota, which will consequently increase uptake of nutrients in the intestinal lumen. Yeast cultures of SCFP have been found to be associated with the promotion of rumen microbial growth (Bach et al., 2007). However, the establishment of bacteria in the gastrointestinal tract depends mainly on the type of diet and

its pH (McCann et al., 2016). In the preweaned period, where calves are experiencing a transition from nursing animals to ruminants, variations on intake and pH could delay the establishment of a mature microbiome and YFP might not be able to counter these effects. Lack of differences in performance might be associated with specific variations within trials, such as immune status of the calves, management, infectious disease incidences, colostrum quality, calf starter nutritional composition, YFP dosages, and length of the study, among others. Prolonged supplementation of YFP until bred could be a better estimate of the beneficial effects of YFP on gut microbiome and performance, considering the establishment of a mature microbiome in the gut at this age. In addition, the dosage of the YFP implemented in this study would not have been enough to exert an effect on performance variables. Yeast fermentation products have been previously shown to exert major positive effects on studies using a challenge model (Brewer et al., 2014; Harris et al., 2017). Our study was a non-challenged trial. In this nonchallenged scenario, the supplementation with two different YFP was not able to improve performance parameters.

Fecal Scores and Fecal Bacteria Count Results

A high incidence of diarrheas, especially during the first 2 wk of life, was presented along the experiment. We evaluated the presence of *Salmonella* and *E. coli* on feces for being two of the most important causes of infectious diarrhea on calves (Cho and Yoon, 2014). Results from *E. coli* and *Salmonella* on feces did not differ between treatments ($P > 0.84$; Table 2). The main objective of measuring their concentration in feces was to confirm that animals were being raised in

Table 2. Determination of fecal presence of *E. coli* and *Salmonella* on samples taken at day 30 from 80 newborn dairy calves fed yeast fermentation products (C, $n = 26$; SCFP, $n = 25$; AOFE, $n = 29$)^a

Item ^b	Treatments ^c			SEM	P-value
	C	AOFE	SCFP		Trt ^d
<i>E. coli</i> , CFU/g	1.91	2.03	1.89	0.33	0.95
<i>Salmonella</i> , CFU/g ^e	0	0	0	—	—

^aData are presented as least square means \pm SEM.

^bCFU = Log¹⁰ of colony-forming units.

^cC = not supplemented; AOFE = 3 g/head/d of LXtract1224 (Biozyme Inc.) in the milk; SCFP = 1 g/head/d of SmartCare (Diamond V) in the milk and 0.7% on DM basis of NutriTek (Diamond V) on the starter feed.

^dTreatment.

^eNo *Salmonella* were found in the fecal samples.

Table 3. Percentage of diarrhea incidence in newborn dairy calves supplemented with yeast fermentation products from birth until weaning at day 56 ± 3 ^a

Item	Treatments ^b			SEM	P-value
	C	AOFE	SCFP		Trt ^c
Diarrhea incidence, % ^d	86	86.5	88	5.9	0.97

^aData are presented as least square means \pm SEM.

^bC = not supplemented; AOFE = 3 g/head/d of LXtract1224 (Biozyme Inc.) in the milk; SCFP = 1 g/head/d of SmartCare (Diamond V) in the milk and 0.7% on DM basis of NutriTek (Diamond V) on the starter feed.

^cTreatment.

^dDiarrhea incidences = Fecal scores were measured daily and scored based on a four-point scale (1 = normal; 2 = soft to loose; 3 = loose to watery; 4 = watery, mucous, and bloody). Animals were considered to have diarrhea and treated with antibiotics when they experienced a fecal score ≥ 3 . Records of scores and number of diarrheic episodes were collected for each calf.

a low-pathogen environment without being challenged with pathogenic bacteria that could influence results. Results also showed no differences between treatments on the incidence of diarrhea ($P = 0.97$; Table 3). Diarrhea and fecal scores are two parameters that have been most affected by the use of YFP (Alugongo et al., 2017). Xiao et al. (2016) demonstrated that supplementation with SCFP increased villus height-to-crypt depth ratio (VCR) of intestinal papillae. A decrease in VCR could lead to osmotic diarrhea caused by malabsorption (Pearson et al., 1978). In that scenario, SCFP's supplementation might improve fecal scores by improving VCR and, with that, nutrient absorption (Xiao et al., 2016). Brewer et al. (2014) challenged 40 dairy calves with *Salmonella enterica* and found that those animals under an SCFP

treatment presented less clinical signs of disease, less incidence of diarrhea and pyrexia, and a detriment on *Salmonella* colonization and shedding. Similarly, Galvão et al. (2005) demonstrated a reduction in days with diarrhea when calves were supplemented with SCFP. Magalhães et al. (2008) also showed differences between *S. cerevisiae*-supplemented calves and controls where supplemented animals experienced lower fecal scores and fewer days with diarrhea. Results from the present study are in accordance with previous research (Lesmeister et al., 2004; Yohe et al., 2015) where supplementation with YFP did not decrease the incidence of diarrhea overall. When consulting different literature, results on the use of YFP over health parameters are inconsistent. These variations could be related to dosages of the YFP used, number of animals enrolled, length of the study, and use of challenged models, among others. In this study, the estimation of diarrhea was performed by following the standard operation protocol of the commercial farm. In this facility, whenever animals presented a fecal score ≥ 3 , they were immediately treated with antibiotics. Blood samples for metabolites, GLP-2, and sugar tests were taken within the first 2 wk of life, which was also the time where most calves experienced diarrhea. For this study, 85.5% of the animals experienced at least one event of diarrhea during the first 2 wk of life. A better estimation of diarrheas could be made by considering a calf to be diarrheic if the diarrhea episode lasts for at least 3 d with a fecal score ≤ 3 as stated in previous research (Araujo et al., 2015). However, in this study, we followed the farm standard operation protocol and treated animals with antibiotics whenever their fecal score was ≤ 3 but without waiting for 3 d as proposed by Araujo et al. (2015). A recent study on dairy calves showed that animals that experience diarrhea in the first weeks of life are born with an increase in their intestinal permeability (Araujo et al., 2015). The reasons for this are not completely understood to date. However, a possible explanation could be based on a study conducted by Wilhelm et al. (2017), where it was shown that female calves born from hypocalcemic cows had greater incidence of diarrhea when compared to those born from nonhypocalcemic cows, demonstrating the influence that prepartum conditions of the dam can exert over newborns' gut health. However, it could be possible that supplementation with YFP might not have an effect on decreasing diarrhea events in animals that are born with this particular condition of leaky gut.

This could also serve as an explanation of why YFP usually shows better response improving gut permeability on challenged trials where the cause of diarrhea is a pathogenic bacteria and not a congenital predisposition.

GLP-2 Results

To our knowledge, this is the first study evaluating the relationship between the use of YFP on plasma GLP-2 concentration and intestinal permeability in dairy calves. GLP-2 is a gastric hormone that is released into circulation after the arrival of nutrients from the diet (Drucker and Yusta, 2014). A previous study showed that supplementation with a prebiotic similar to YFP was able to increase intestinal proglucagon mRNA and, as a result, plasma GLP-2 plasma concentration (Cani et al., 2009). Therefore, we assumed that the use of YFP might increase plasma GLP-2 concentration in supplemented calves. The reason for the increase in plasma GLP-2 plasma concentration after YFP consumption is associated to the development of a selective microbiota in the gut controlling, which might increase this peptide synthesis and secretion (Cani et al., 2009). Results from this study did not show differences between treatments for plasma concentration of total GLP-2 ($P = 0.78$; Table 4). Cani et al. (2009) fed a prebiotic with similar functions to YFP to obese mice for 4 wk and found that YFP supplementation increased plasma GLP-2 concentration and proglucagon (GLP-2 precursor) mRNA expression in treated mice. Conclusions from Cani et al. (2009) stated that specific changes in gut microbiota increased GLP-2 concentration. A different

study showed that, when supplemented with SCFP, dairy calves exhibited an increase in their intestinal papillae length, papillae width, and papillae surface area (Xiao et al., 2016). These changes in intestinal morphology are positively correlated with intestinal absorption of nutrients. Similar results were also found in piglets (Shen et al., 2009) and broilers (Gao et al., 2008). Brewer et al. (2014) found that, during an experimental infection with *Salmonella*, dairy calves supplemented with SCFP had fewer adhesions of bacteria in the ileum, corresponding with a lower bacterial shedding in feces. These results serve as a basis for the understanding of the beneficial effects of YFP over intestinal health. Additionally, a healthier gut would be able to absorb nutrients in a more efficient way, stimulating the release of this gastrointestinal hormone. Under physiological conditions, active GLP-2 (1-33) is inactivated after cleavage with dipeptidyl-peptidase VI and converted to its truncated form GLP-2 (3-33), which is considered to be biologically inactive. In the present study, the assay used to measure plasma GLP-2 concentration was not able to discriminate between active and inactive forms. Therefore, results are an expression of total plasma GLP-2 concentration. Measurement of only active GLP-2 would be a better estimate of this hormone's concentration (Hartmann et al., 2000). However, to date, there is one source of the specific antibody to measure active GLP-2 (1-33; J. J. Holst, Panum Institute, Copenhagen, Denmark, antibody no. 92160; Hartmann et al., 2000) but we did not have access to it. The understanding of GLP-2 secretion stimulus and the effect that YFP supplementation can have over it needs to be further investigated.

Table 4. The effects of supplementing newborn dairy calves with yeast fermentation products on plasma concentrations of glucose, NEFA, insulin, IL-1 β , and GLP-2^a

Item	Day	Treatments ²			SEM	P-value		
		C	AOFE	SCFP		Trt ³	Day	Trt \times day
Glucose, mg/dL	7	109.2	112.1	114.2	2.96	0.21	<0.01	0.17
	14	100.2	108.2	101.2				
NEFA, mM	7	285.7	253.7	226.3	17.09	0.44	<0.01	0.06
	14	218.8	213.3	226.9				
Insulin, uU/mL	7	32.2	31.5	37.8	6.19	0.95	0.12	0.64
	14	40.7	43.5	39.4				
IL-1 β , pg/mL	14	15.7 ^a	23.6 ^{ab}	32.1 ^b	4.51	0.06	—	—
GLP-2, pg/mL	14	402.1	357.2	359.9	49.65	0.78	—	—

Measurements from blood samples taken on days 7 and 14 of the trial.

^aData are presented as least square means \pm SEM.

^bC = not supplemented; AOFE = 3 g/head/d of LXtract1224 (Biozyme Inc.) in the milk; SCFP = 1 g/head/d of SmartCare (Diamond V) in the milk and 0.7% on DM basis of NutriTek (Diamond V) on the starter feed.

^cTreatment.

^{ab}Mean values with different superscript letters within a column indicate a difference ($P \leq 0.05$).

Plasma Variables Results

Plasma concentration of glucose, NEFA, and insulin can be utilized as indicators of energy metabolism in calves. An animal with increased gut permeability will have an increase in its energy expenditure (due to, i.e., costs of immune response activation, loss of nutrients in feces, and tissue reparation, among others); therefore, the use of YFP could potentially impact plasma glucose, NEFA, and insulin concentrations by decreasing gut permeability. No differences were found between treatments for plasma glucose or insulin concentration ($P > 0.1$; Table 4). Insulin is one of the most important hormones regulating energy metabolism in ruminants. One of its functions is to stimulate glucose uptake by tissues. The lack of differences in insulin concentration is in accordance with the lack of differences in glucose (Table 4) concentration after YFP supplementation. Possible explanations could be that the dose of YFP supplemented was not enough to generate a noticeable change in these metabolites, the need for some level of challenge in order to generate a beneficial effect. Also, under conditions of restricted feeding, such as artificial rearing, animals may be unable to increase their growth or metabolism to the point of showing differences in these metabolites. To our knowledge, only one study conducted on calves found differences in glucose concentration after supplementation of YFP based on SCFP (Galvão et al., 2005). Results on metabolites' concentrations are also related to results on performance. Starter intake did not differ between treatments; therefore, it might explain the lack of differences in plasma glucose and insulin concentrations. There was a time by treatment tendency for NEFA ($P = 0.06$) plasma concentrations (Table 4), where NEFA concentration tended to increase on day 7 for C and AOFE and decreased later on day 14. The concentration of NEFA for SCFP remained constant for days 7 and 14. There was a difference on day 7 for NEFA concentration, being 286 and 226.19 mM for C and AOFE, respectively. Supplementation of AOFE showed a more constant NEFA concentration. Finally, there was a tendency for treatments on plasma IL-1 β ($P = 0.06$; Table 4). This tendency was due to differences found between C and SCFP, which had a plasma IL-1 β concentration of 15.71 and 32.01 pg/mL, respectively, while AOFE had an intermediate concentration of 23.6 pg/mL. One suggested mode of action of YFP is via competition of β -glucans and mannan-oligosaccharides (contained in the yeast cell wall of the YFP) against

pathogenic microorganisms for binding sites in the gut wall (Alugongo et al., 2017). This competition can potentially decrease attachment of bacteria to the intestinal epithelium preventing future inflammatory processes mediated by proinflammatory IL-1 β . In addition, one of the recognized effects of GLP-2 is its capacity to decrease proinflammatory cytokines and to increase anti-inflammatory IL-10 (Connor et al., 2015). Cani et al. (2009) showed that changes in gut microbiota after supplementation with a product, similar to the YFP used in this study, decreased cytokines and chemokines concentrations. Several studies have shown a decrease in TNF- α (Moran et al., 2012), serum amyloid A (Connor et al., 2017), and tyrosine nitrated proteins (Connor et al., 2013) concentrations when supplementing YFP. A decrease in plasma IL-1 β concentration due to YFP supplementation was expected considering a potential beneficial effect linked to GLP-2 anti-inflammatory effects (Cani et al., 2009). However, in this study, GLP-2 concentration was not increased by YFP supplementation. Therefore, the increase in plasma IL-1 β concentration for SCFP when compared to C cannot be explained based on GLP-2 concentration. Also, we were expecting that YFP would decrease IL-1 β concentration; however, we did not find that result and, based on previous literature (Cani et al., 2009; Moran et al., 2012), we cannot explain the physiological mechanism for that increase.

Intestinal Permeability Results

Assessment of intestinal integrity was conducted via the lactulose and D-mannitol test. The principle of this test is based on the capacity that these saccharides have to cross the intestinal layer via transcellular and paracellular routes. Lactulose is a large-size disaccharide that, in normal physiological conditions, is not absorbed in the small intestine but fermented later by bacteria in the large intestine (Bischoff et al., 2014). When the integrity of the small intestine is compromised, lactulose will cross the intestinal layer via the paracellular route (Bischoff et al., 2014). D-mannitol is a small-size monosaccharide that is normally absorbed in the small intestine via transcellular pathway without significant metabolism (Wang et al., 2015). In a leaky gut situation, both markers can be found in plasma or urine. For this study, sugars were measured from plasma samples for being a more practical method than urine collection, particularly, considering that this study was conducted at a commercial farm. The final assessment of

intestinal integrity is made by calculating the ratio between both sugars' concentrations in plasma. This ratio will reflect the functionality of the paracellular pathway (Bischoff et al., 2014). Therefore, a low serum lactulose:D-mannitol ratio would reflect a normal intestinal layer, while a high serum lactulose:D-mannitol ratio would be indicative of a failure on the intestinal integrity (Hall, 1999). Results from the present study showed no differences in plasma lactulose and D-mannitol concentrations or their ratio ($P = 0.6$; Table 5). We assumed that an increase in GLP-2 concentration will stimulate cell proliferation and tight junction proteins' expression, which would favor intestinal mucosa integrity, decreasing pathogens' translocation and finally decreasing the incidence of diarrhea. GLP-2 has also been shown to increase nutrients' absorption and transport via increased expression of intestinal transporters and enzymes (Connor et al., 2015). In addition, GLP-2 supplementation was probed to increase intestinal weight

and epithelial cells proliferation in the jejunum of newborn dairy calves (Connor et al., 2013) and to improve gut integrity by increasing the expression of the tight junction proteins occluding and ZO-1 (Walker et al., 2015). However, we did not find any differences in GLP-2 concentration that could justify a decrease in gut permeability and, with that, a decrease in diarrhea incidence. There is evidence that some calves might have greater gut permeability right after they are born (Araujo et al., 2015). Considering this evidence, a gut permeability test right after birth, and the selection of only those animals who show a negative permeability response, would be a better approach on the effects of GLP-2 on gut permeability.

Regression Analysis

Results showed that SI at day 14 has a negative association with the sugars' ratio; in other words, the sugars' ratio decreases 0.2 pmol/uL with every increase of 1 g of SI ($P = 0.03$; Table 6). This could be explained as higher blood flow in the gut due to the arrival of nutrients (showed by the increase in SI) and these nutrients' absorption could be facilitated by an increase in gut permeability (showed by the increase in sugars' ratio). Plasma IL-1 β tended to be positively associated with the sugars' ratio. Sugars' ratio tended to increase 0.0028 pmol/uL with every increase of 1 pg/mL of IL-1 β ($P = 0.09$; Table 6). Plasma IL-1 β could potentially increase gut permeability. IL-1 β has been shown to increase tight junction permeability of Caco-2 cells in an NF- κ B activation-mediated process (Al-Sadi and Ma, 2007). IL-1 β was also proved to cause down-regulation of the transmembrane protein occluding expression, a decrease in its mRNA expression, and alterations in its tight junctional localization (Al-Sadi and Ma, 2007). Results from Ig G disagree with those from Araujo et al. (2015) showing a positive association of Ig G with the sugars' ratio. The sugars' ratio tended to increase 0.0037 pmol/uL with every increase of 1 mg/dL of Ig G ($P < 0.01$;

Table 5. The effects of supplementing newborn dairy calves with yeast fermentation products on plasma lactulose and D-mannitol concentration measured at day 14 of age^a

Item ^b	Treatments ^c			SEM	P-value
	C	AOFE	SCFP		
Plasma lactulose, pmol/uL	22.9	25.9	25.5	2.12	0.60
Plasma D-mannitol, pmol/uL	14.9	16.2	15.7	1.10	0.70
Plasma lactulose:D-mannitol	1.6	1.6	1.6	0.07	0.75

^aData are presented as a least square means \pm standard error of the mean (SEM).

^bLactulose and D-mannitol were used as gut permeability markers. On day 14, lactulose and D-mannitol sugars were added into the buckets and mixed with the morning milk at a concentration of 21 g/31.5 mL and 133.3 g/31.5 mL, respectively. Blood samples were taken an hour after markers were consumed with the milk.

^cC = not supplemented; AOFE = 3 g/head/d of LXtract1224 (Biozyme Inc.) in the milk; SCFP = 1 g/head/d of SmartCare (Diamond V) in the milk and 0.7% on DM basis of NutriTek (Diamond V) on the starter feed.

Table 6. Regression analysis between plasma concentration of IL-1 β , Ig G, and sugars' ratio (lactulose:D-mannitol), starter intake at day 14, and ADG at day 14^a

Variable ^a	Estimate \pm SEM			
	Intercept	SI, kg	IL-1 β , pg/mL	Ig G, mg/dL
Sugars' ratio	-6.4 \pm 2.93*	-0.2 \pm 0.09*	0.0028 \pm 0.0016 \dagger	0.0037 \pm 0.0013**
ADG, kg	0.57 \pm 0.051**	0.08 \pm 0.025**	—	—

^aThe full models are sugars' ratio = SI at day 14 + IL-1 β + plasma Ig G concentration + plasma GLP-2 concentration; and ADG at day 14 = SI + sugar ratio + plasma insulin + plasma Ig G concentration + incidence of diarrhea at the sampling time. Only the variables that are significant ($P < 0.1$) are presented on the table (\pm SEM).

* $P \leq 0.05$. $\dagger P > 0.05$ and $P \leq 0.10$.

Table 6). Calves that were born with an increase in their intestinal permeability (higher sugars' ratio) will potentially be more efficient absorbing Ig G. That increase in their intestinal permeability might remain until day 14. In relation to the results for ADG at day 14, only SI at day 14, as expected, showed a positive association. Plasma Ig G, insulin concentration, the incidence of diarrhea at the sampling time, and sugars' ratio did not show an association with ADG at day 14.

CONCLUSIONS

We did not observe improvements on performance, intestinal permeability, or plasma concentration of GLP-2 on dairy calves supplemented with YFP during the preweaned stage. In our study, plasma GLP-2 concentration did not show an association with gut permeability, whereas IL-1 β concentration had a positive tendency with it.

Under the current conditions, YFP were not able to increase performance or gut permeability. A challenged model might be more suitable to study the effects of YFP supplements on these parameters. Further research needs to be conducted on the mechanism of action of these YFP to completely understand their physiological responses on newborn calves.

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