

Effects of Flavonoids from Mulberry Leaves and *Candida tropicalis* on Performance and Nutrient Digestibility in Calves

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Abstract

Flavonoids from mulberry leave (FML) are natural flavonoids, and *Candida tropicalis* (CT) is yeast like microbe. In this study, forty-eight male calves were selected with born weight (40.5±0.7 kg) and 20±2 days age, divided into 4 groups randomly. FML was supplemented in a dairy calf starter at 2 g/d per calf before weaning, or 4 g/d per calf after weaning (FML group), while CT was added in a dairy calf starter at 1 g/d per calf (CT group). Our results showed that FML could be used to enhance body weight (BW) of calves through enhancing apparent digestibility of ether extract (EE) of the diet, and increased the levels of serum growth hormone (GH) and insulin-like growth factors 1 (IGF-1) in calves after the age of 56 days. CT enhanced the BW of calves before weaning through increasing the apparent digestibility of neutral detergent fiber (NDF) of the diet, and elevated the apparent digestibility of EE of the diet of calves after weaning through increasing the level of serum IGF-1 in calves. Furthermore, the mixture of FML and CT plays a synergistic role in enhancing growth, improving feed intake and nutrient digestibility. In conclusion, FML and CT could be used as additives to increase growth and nutrient digestibility in calves.

Keywords: Calves, Flavonoids from mulberry leave, *Candida tropicalis*, Nutrient digestibility, Hormone level

Dut Yaprağından Elde Edilen Flavonoidler İle *Candida tropicalis*'in Buzağılarda Performans ve Besin Sindirilebilirliği Üzerine Etkileri

Özet

Dut yaprağından elde edilen flavonoidler (FML) doğal flavonoidler olup *Candida tropicalis* (CT) de mantar benzeri mikroplardır. Bu çalışmada, doğumda 40.5±0.7 kg ağırlığa sahip 20±2 günlük kırk sekiz erkek buzağı kullanılarak rastgele 4 gruba ayrıldı. FML sütten kesme öncesi her bir buzağı için 2 g/d olarak buzağı başlangıç yemi içerisinde veya her bir buzağı için 4 g/d olarak sütten kesme sonrası (FML grup), CT ise her bir buzağı için 1 g/d olarak buzağı başlangıç yemi içerisinde (CT grup) verildi. Çalışma sonucunda FML'nin diyetin eter ekstraktının (EE) sindirilebilirliğini ve serum büyüme hormonu (GH) ve insülin benzeri büyüme faktörü 1 (IGF-1)'in seviyelerini belirgin bir şekilde arttırmak yoluyla 56 günlükten sonrasında buzağuların vücut ağırlığını (BW) geliştirmek amacıyla kullanılabileceği tespit edilmiştir. CT; buzağuların vücut ağırlığını sütten kesme öncesinde diyetteki nötr deterjan fiberin sindirilebilirliğini belirgin ölçüde geliştirdi ve serum IGF-1'in seviyesini yükseltmek suretiyle sütten kesme sonrasında diyetteki eter ekstraktının sindirilebilirliğini belirgin ölçüde artırdı. FML ve CT karışımı büyümeyi geliştirme, gıda tüketimini ve besin sindirilebilirliğini iyileştirmekte sinerjistik bir rol oynadı. Sonuç olarak, FML ve CT büyüme ve besin sindirilebilirliğini arttırmak amacıyla buzağılarda bir katkı olarak kullanılabilir.

Anahtar sözcükler: Buzağı, Dut Yaprağı Flavonoidleri, *Candida tropicalis*, Besin sindirilebilirliği, Hormon seviyesi

INTRODUCTION

It is well documented that there are a number of potential risks for human health in using antibiotics in food-producing animals, including drug residues in meat products, increasing bacterial resistance and environmental contamination^[1]. Flavonoids are found in berries, tea,

cocoa, soybeans, grains, and plant leaves, are a class of organic polyphenolic compounds^[2]. It is through various mechanisms including protection against oxidative stress, and preservation of epithelial barrier function and immunomodulatory properties that flavonoids are used in acute or chronic intestinal inflammation^[3]. Dietary flavonoids (quercetin and morin) have marked effects on the fatty



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acid composition of blood plasma, liver, or breast muscle lipids in vitamin E-deficient chicks [4]. Condensed tannins (a polyflavonoid) can mitigate methane emission by feeding *Leucaena leucocephala* in sheep [5]. It is effective in improving rumen fermentation and reducing the incidence of rumen acidosis through supplementation of natural flavonoids extract from bitter orange and grapefruit [6]. Sainfoin which contains rich condensed tannins can increase beneficial fatty acids and reduce skatole content in lamb meat [7]. Flavonoids from mulberry leaves (FML) have positive effects on the hypoglycaemic, antihypercholesterolemic and anti-oxidative potential in rats [8].

Probiotics are live microorganisms including bacteria and yeasts, which can improve intestinal health and immune response, prevent acute and antibiotic-associated diarrhea [9]. It is reported that GSY10 is the most promising oleaginous yeast for microbial lipid production from molasses, and it can be used as feed supplement for microbial lipid production in dairy cattle [10]. The yeast culture extract can activate natural killer (NK) cells and B lymphocytes *in vitro*, which plays a role in the anti-inflammatory effects [11]. Yeast-based immunogen (EpiCor) possesses conspicuous anti-inflammatory activity, and can directly induce activation of chemotactic awareness of lymphocyte subsets *in vitro* [12]. After experimental challenged with *Salmonella*, pre-weaned dairy cows fed with *Saccharomyces cerevisiae* fermentation products have fewer bouts of diarrhea and fever, more beneficial microbe in rumens and higher weight gain comparing with no-fed group [13]. *Lactic acid bacteria* or *Bacillus* species generally target the lower intestine to stabilize the gut microbiota, which decreases the risk of pathogen colonization in young ruminants [14]. As a fungal organism, *Candida tropicalis* (CT) can grow as yeast morphology [15]. It is known that the somatotrophic axis primarily consists of growth hormone (GH), insulin-like growth factors (IGF), as well as their associated carrier proteins and receptors, which plays a key role in the control of the protein anabolism, fat deposition, and growth rate in animals [16]. It was hypothesized that supplementation of FML and CT in starter of calves might improve consumption of nutriment, accelerate animal growth, and feed intake. Thereby, the aim of current study was to determine the effects of supplementation FML and CT in the starter on growth, performance and the concentrations of GH and IGF-1 in plasma during the first 80 days of age in calves.

MATERIALS and METHODS

Materials

The extract of FML was purchased from Xi'an Feida Biotechnology Co. Ltd., and there were 50 mg of FML per g of extract, which was analyzed by the manufacturer. CT was from Beijing Vano Biological Engineering Co., Ltd., and the concentration of live bacteria was 5×10^9 CFU/g, which was provided by the manufacturer. The milk replacer is

provided by Beijing Jingzhun Animal Nutrition Center, and the starter is from Beijing Sanyuan Luhe Feed Factory. The basal diet consists of milk replacer and starter with no antibiotics and microbial preparation. The ingredients and nutrient levels of basal diet are shown in Table 1.

Animals and Experimental Design

Holstein male calves were managed on the first farm of Western Suburbs, Beijing Sanyuan Luhe Cow Breeding Center. The experimental protocol was approved by the Chinese Academy of Agricultural Sciences Animal Ethical Committee, and humane animal care and handling procedures were followed throughout the experiment. Forty-eight male calves with 20 ± 2 days old, and 40.5 ± 0.7 kg birth weight were selected from cows with natural childbirth and between 3 and 5 years old. The calves were fed adequate colostrum during the first 3 days and then fed milk replacer until 80 days of age. Calves randomly were divided into 4 groups (n=12) based on parity and birth weight. The control group (Ctrl group) was fed with basal diet, while the other three groups were added with FML (FML group, 2 g/d per calf before weaning, or 4 g/d per calf after weaning), with CT (CT group, 1 g/d per calf) and with the above two additives (FML + CT group, FML

Table 1. Nutrient composition and levels of basal diet (air-dry basis) %

| Items | Starter | Milk Replacer |
|-------------------------------|---------|---------------|
| Corn | 20.0 | |
| Extrude corn | 22.9 | |
| Soybean meal | 20.0 | |
| Extruded soybean | 18.0 | |
| Whey powder | 5.00 | |
| Wheat bran | 10.0 | - |
| CaHPO ₄ | 0.800 | |
| Limestone | 1.80 | |
| Salt | 0.500 | |
| Premix* | 1.00 | |
| Total | 100 | |
| Nutrient levels | | |
| DM (Dry matter) | 85.4 | 95.4 |
| OM (Organic matter) | 92.2 | 94.9 |
| CP (Crude protein) | 19.1 | 24.3 |
| EE (Ether extract) | 2.21 | 12.9 |
| NDF (Neutral detergent fiber) | 18.6 | 4.02 |
| ADF (Acid detergent fiber) | 10.7 | 2.11 |
| Ca | 1.09 | 1.07 |
| P | 0.473 | 0.482 |
| GE (Gross energy) MJ/kg | 15.5 | 19.9 |

* Premix supplemented with VA 15.000 IU, VD 5.000 IU, VE 50 mg, Fe 90 mg, Cu 12.5 mg, Mn 30 mg, Zn 90 mg, Se 0.3 mg, I 1.0 mg and Co 0.5 mg for per kg starter

2 g/d + CT 1 g/d per calf before weaning, or FML 4 g/d + CT 1 g/d per calf after weaning). The experiment began at 21 days old and lasting 60 days. At the age of 55 days old, calves were weaned. The diet for calves contained milk replacer and starter. Milk replacer was offered daily at 10% of body weight (BW) (adjusted weekly) and starter was offered *ad libitum* throughout the 60 days trial period. Each calf was housed in an individual hutch during the whole experiment period except the metabolic study period during which each calf was raised in an individual metabolic cage. There are two metabolic trials in the whole experiment period. One trial began at the age of 43 days lasting 5 days (preweaning), the other began at the age of 60 days lasting 5 days too (postweaning). BW was measured at 21, 28, 42, 56 and 80 days old during the whole trial period, and starter intake was recorded daily.

Sample Collection and Analysis

Every three calves that were from the correspond group, and reached the average BW were selected for the metabolic trial. Urinary and fecal excretions of every calf (4 groups in all, one group having 3 calves) were entirely collected daily for analyzing the apparent digestibility of dry matter (DM), ether extract (EE), neutral detergent fiber (NDF) and acid detergent fiber (ADF). The mixed sample was from 10% of total amount of feces, and 10 g mixed fecal sample was treated with 10 mL of 10% dilute hydrochloric acid for nitrogen fixation. Then a 500-g feces sample was taken, dried at 103°C for 48 h, ground in a Cyclotec 1093 mill (Tecator, Sweden). The digestion rate was calculated as previously described [17]. The blood samples (jugular venipuncture) were collected before the morning feeding at 28, 42, 56 and 80 days old, respectively. Plasma samples were stored at -19°C after centrifugation (3000×g, for 15 min, at 25°C) for analysis of GH and IGF-1 by radioimmunoassay (RIA) as previously described [18]. IGF-1 antibody (sc-1422, Santa Cruz Biotech, CA) was used to analyze the IGF-1 concentration, and the GH concentration was determined using an antibody (sc-10365, Santa Cruz Biotech, CA).

Statistical Analysis

The experimental design was a randomized complete block design. Continuous variables were analyzed by ANOVA using the MIXED procedure of SAS (SAS Inst. Inc., Cary, NC, 2003). The model included fixed effect and the random effect. Treatments, days (as a repeated effect), and their interaction were as the fixed effects, and the calf was as the random effects. Restricted Maximum Likelihood was used to estimate least square mean values. Where treatment effects were significant the means were analyzed using Tukey's procedure for multiple comparisons. The initial BWs were modeled as a Covariate to further control the experimental error. Differences were considered statistically significant at the 95% confidence level ($P < 0.05$).

RESULTS

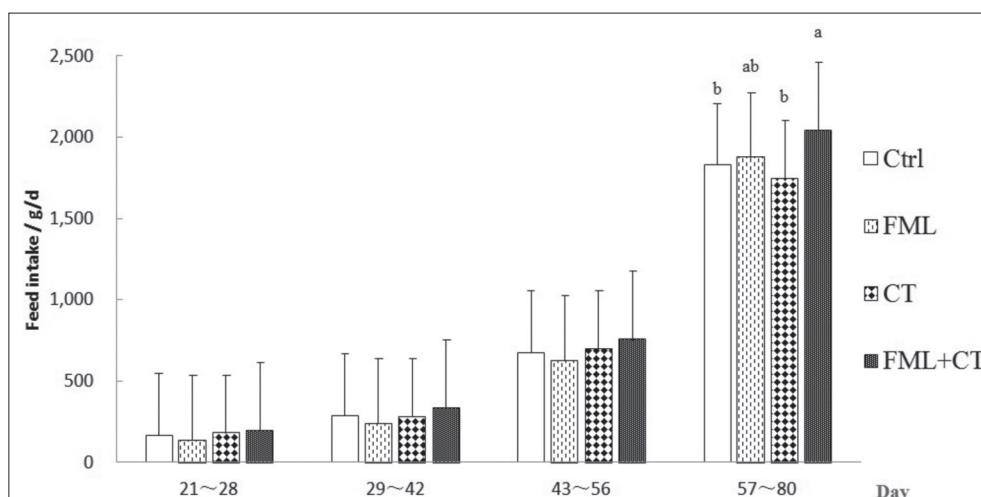
The Effect of FML on Performance and Plasma Hormone Level

It was shown in Fig. 2 that the BW of FML group was significantly higher ($P < 0.05$) than that of Ctrl group after weaning, but there was no significant effect of FML on BW before weaning. It was observed in Fig. 1 that there was no significant effect of FML on starter intake throughout the experiment in calves ($P < 0.05$). Meanwhile significant changes ($P < 0.05$) were observed between FML and Ctrl group in the levels of plasma GH and IGF-1 at the age of 80 days (Table 3), and the digestibility of EE in FML group was significantly higher ($P < 0.05$) than that in Ctrl group after weaning (Table 2).

The Effect of CT on Performance and Plasma Hormone Level

The diet supplementation with CT did not affect starter intake ($P > 0.05$) throughout the experiment in calves (Fig. 1), but the BW (Fig. 2) of calves of CT group was significantly higher than that of Ctrl group only at the age of 56 days ($P < 0.05$), while the *apparent digestibility* of NDF

Fig 1. Effect of flavonoids from mulberry leaves and *Candida tropicalis* on feed intake. Ctrl, no additive; FML, FML 2 g/d per calf before weaning, or FML 4 g/d per calf after weaning; CT, *Candida tropicalis* (1 g/d per calf); FML+CT, FML (FML 2 g/d per calf before weaning, or FML 4 g/d per calf after weaning) + *Candida tropicalis* (1 g/d per calf). Within the same row with different superscripts indicated significant differences ($P < 0.05$)



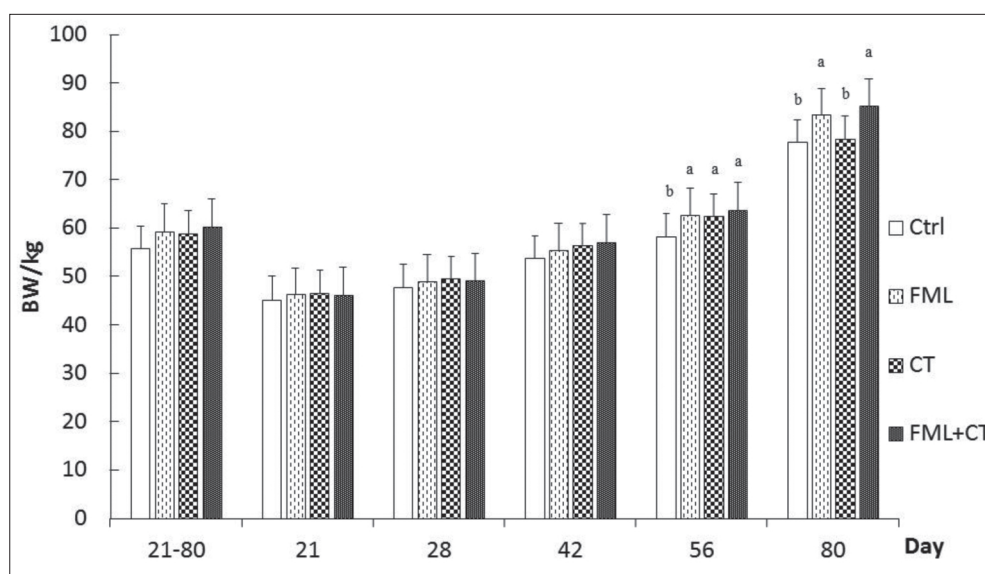


Fig 2. Effect of flavonoids from mulberry leaves and *Candida tropicalis* on body weight. Ctrl, no additive; FML, FML 2 g/d per calf before weaning, or FML 4 g/d per calf after weaning; CT, *Candida tropicalis* (1 g/d per calf); FML + CT, FML (FML 2 g/d per calf before weaning, or FML 4 g/d per calf after weaning) + *Candida tropicalis* (1 g/d per calf). Within the same row with different superscripts indicated significant differences ($P < 0.05$)

Table 2. Effect of flavonoids from mulberry leaves and *Candida tropicalis* on nutrient digestibility

| Items | Treatment | | | | SEM | P-value |
|---------------------------------|-------------------|--------------------|-------------------|-------------------|-------|---------|
| | Ctrl | FML | CT | FML+CT | | |
| Pre-weaning male calves | | | | | | |
| Dry matter | 79.3 | 79.2 | 79.0 | 80.1 | 1.09 | 0.976 |
| Organic matter | 81.3 | 80.5 | 80.6 | 81.5 | 1.07 | 0.883 |
| Ether extract | 79.3 | 75.8 | 79.1 | 69.5 | 2.55 | 0.905 |
| Neutral detergent fiber | 19.2 ^b | 21.3 ^b | 25.7 ^a | 19.8 ^b | 0.983 | 0.163 |
| Acid detergent fiber | 27.4 | 29.6 | 27.7 | 29.4 | 1.62 | 0.0994 |
| Post-weaning male calves | | | | | | |
| Dry matter | 79.3 | 84.7 | 81.6 | 84.9 | 1.52 | 0.342 |
| Organic matter | 81.3 | 86.4 | 82.2 | 86.9 | 1.34 | 0.328 |
| Ether extract | 33.7 ^b | 53.5 ^a | 46.3 ^a | 62.7 ^a | 3.16 | 0.0317 |
| Neutral detergent fiber | 49.3 ^b | 57.5 ^{ab} | 48.3 ^b | 60.7 ^a | 2.03 | 0.0287 |
| Acid detergent fiber | 63.7 ^b | 66.3 ^{ab} | 65.4 ^b | 72.5 ^a | 1.36 | 0.0322 |

Ctrl, no additive; FML, FML 2 g/d per calf before weaning, or FML 4 g/d per calf after weaning; CT, *Candida tropicalis* (1 g/d per calf); FML+CT, FML (FML 2 g/d per calf before weaning, or FML 4 g/d per calf after weaning) + *Candida tropicalis* (1 g/d per calf). Within the same row with different superscripts indicated significant differences ($P < 0.05$)

of CT group was significantly higher than that of Ctrl group before weaning ($P < 0.05$). The digestibility of EE in CT group was significantly higher ($P < 0.05$) than that in Ctrl group after weaning (Table 2), while the level of plasma IGF-1 in CT group was obviously higher ($P < 0.05$) than that in Ctrl group post weaning (Table 3).

The Effect of FML + CT on Performance and Plasma Hormone Level

The results of current study (Fig. 1, Fig. 2) indicated that BW and starter feed in FML + CT group were significantly higher ($P < 0.05$) than that in Ctrl group at the age of 57-80 days. There was no significant change in BW between FML + CT and Ctrl group at the age of 21-42 days ($P > 0.05$). The levels of plasma GH and IGF-1 in FML + CT group

were significantly higher ($P < 0.05$) than that in Ctrl group at the age of 80 days (Table 3). The diet supplementation with FML+CT simultaneously enhanced digestibility of EE, NDF and ADF ($P < 0.05$) significantly compared with that no supplementation after weaning in calves (Table 2).

DISCUSSION

The use of antibiotics for growth promotion has been totally banned in many countries, owing to drug residues in meat products and increasing bacterial resistance by use and misuse of in-feed antibiotics in food-producing animals [19], so utilization of phytochemicals in feed for food animal production has good potential [20]. It is fed flavonoids extracted from propolis that calves have

Table 3. Effect of flavonoids from mulberry leaves and *Candida tropicalis* on GH and IGF-1

| Items | Treatment | | | | SEM | P-value | | |
|----------------------|-------------------|-------------------|--------------------|-------------------|--------|---------------|-----------------|-------|
| | Ctrl | FML | CT | FML+CT | | Treatment age | Treatment × Age | |
| GH (ng/mL) | | | | | | | | |
| 28-80 | 3.21 | 3.31 | 3.33 | 3.39 | 0.0526 | 0.613 | 0.00157 | 0.169 |
| 28 | 3.28 | 2.95 | 3.02 | 3.09 | 0.0618 | 0.645 | - | - |
| 42 | 3.23 | 3.18 | 3.25 | 3.16 | 0.0578 | 0.463 | | |
| 56 | 3.25 | 3.43 | 3.67 | 3.71 | 0.293 | 0.0681 | | |
| 80 | 3.23 ^b | 3.72 ^a | 3.31 ^{ab} | 3.75 ^a | 0.181 | 0.0357 | | |
| IGF-1 (ng/mL) | | | | | | | | |
| 28-80 | 162 | 225 | 211 | 221 | 9.35 | 0.0452 | 0.0132 | 0.246 |
| 28 | 173 | 171 | 206 | 189 | 13.2 | 0.383 | - | - |
| 42 | 167 | 195 | 194 | 158 | 13.5 | 0.336 | | |
| 56 | 159 | 215 | 209 | 214 | 19.5 | 0.0543 | | |
| 80 | 177 ^b | 295 ^a | 277 ^a | 294 ^a | 20.60 | 0.00219 | | |

Ctrl, no additive; FML, FML 2 g/d per calf before weaning, or FML 4 g/d per calf after weaning; CT, *Candida tropicalis* (1 g/d per calf); FML+CT, FML (FML 2 g/d per calf before weaning, or FML 4 g/d per calf after weaning) + *Candida tropicalis* (1 g/d per calf). Within the same row with different superscripts indicated significant differences ($P < 0.05$)

higher BW than those fed no flavonoids until 120 days of age [21]. Results of our present study revealed that calves supplement with FML in the diet have higher BW than those fed no flavonoids after the age of 56 days, without significant difference in feed intake comparing to those fed no flavonoids, which is consistent with the previous reports. Flavonoids have beneficial effects on urinary tract infections, cognitive function and age-related cognitive decline, cancer and cardiovascular disease in human [22], and flavonoids and their metabolites modulated the expression and activity of several metabolic key enzymes, and are involved in regulation of lipid and carbohydrate metabolism [23]. Therefore, the higher BW in FML group may be due to its beneficial effects on several metabolic key enzymes.

Our results showed that the *apparent digestibility* of ADF and NDF was not affected by FML. It was also reported that feeding quebracho tannin extract, a diverse group of polymeric flavonoids, had no effect on ADF and NDF digestibility in Angus heifers [24], which was consistent with our results. However, there was significant effect on the EE of the diet of calves by supplementation with FML comparing Ctrl group after weaning in this study. It was reported that condensed tannins altered ruminal biohydrogenation process of unsaturated fatty acids [25], and the greater digestibility of EE in the FML group than that in Ctrl group may be due to the antioxidant capacities of FML after weaning.

Our results also indicated that there were higher levels of serum GH and IGF-1 in FML group comparing with that in Ctrl group at the age of 80 days. The binding of Genistein to estrogen receptors in the hypothalamus influences the production of GH and growth factors (GF), which lead to

increasing the uterine weight, uterine wall thickness and ovarian weight in Sprague Dawley rats [26]. It is reported that flavanone 8-prenylnaringenin, as a phytoestrogen, increases serum GH, but decreases serum IGF-1 levels in rats [27], and IGF-1 has negative effect on GH gene expression in somatotroph cell line [28]. However, GH can strongly stimulate production of IGF-1 *in vivo*. Many tissues and cells can produce IGF-1, and IGF-1 is mainly secreted by the liver under the control of GH, meanwhile have effects on growth and development mediated partly by the effects of GH [29]. Our results may suggest that FML promotes the growth and development of calves by increasing the levels of serum GH and IGF-1. Therefore, FML enhanced apparent digestibility of EE of the diet, and increased the levels of serum GH and IGF-1 in calves, which led to the increased BW after the age of 56 days by supplemented with FML.

It is reported that yeast culture can enhance crude protein and cell wall digestibility, ruminal molar proportion of propionate and plasma glucose concentration in Baluchi lambs [30], and Jersey calves feed live yeast product have greater final BW at 63 days than calves fed none [31]. Our results indicated that there was significant effect on BW of calves supplemented with CT comparing with that no CT only at the age of 56 days, with no significantly difference in the feed intake during total experimental stage. Meanwhile the apparent digestibility of NDF of CT group was significantly higher than that of Ctrl group before weaning. Lesmeister *et al.* [32] reported that Holstein calves fed 2% yeast culture had greater BW at 42 days of age than calves with receiving no yeast. There are improvements in grain intake, BW gain, and blood parameters of calves when fed live yeast only during the pre-weaning period [33]. The calves fed *Saccharomyces cerevisiae* have greater BW during the

pre-weaning period, because yeast can improve growth and activity of fiber-degrading bacteria and fungi, stabilize rumen pH, prevent lactate accumulation, improve ruminal microbial colonization, and set up fermentative processes^[34], which is consistent with our study. Our study suggested that CT enhanced the BW of calves before weaning through manipulating rumen fermentation and increasing the apparent digestibility of NDF of the diet.

Furthermore, the results of present study showed that the apparent digestibility of EE in CT group was significantly higher than that in Ctrl group after weaning, and CT have significant effect on the level of serum IGF-1 comparing with Ctrl group in post-weaning male calves. It is supplemented with *Lactobacillus plantarum* that serum IGF-1 can return to pre-challenge values by day 13 post-challenge orally with *Salmonella* in pigs^[35]. It is treated with *Lactobacillus rhamnosus* that Zebrafish exhibits a high gene expression level for IGF-1 comparing to untreated group at 6 days post fertilization^[36]. Therefore, CT enhanced the apparent digestibility of EE of the diet in calves after weaning through increasing the level of IGF-1 and manipulating rumen fermentation.

Results of the current experiment showed that mixture of FML and CT plays a synergistic role in enhancing growth, improving feed intake and nutriment *digestibility*. It achieved the best effect among four treatments through simultaneously supplementation the two additives (FML + CT) to the starter of calves. BW, starter feed and the levels of plasma GH and IGF-1 were observed significantly higher ($P<0.05$) in FML + CT group compared with Ctrl group at the age of 80 days. Furthermore, the *digestibility* of EE, NDF and ADF in FML + CT group was significantly higher ($P<0.05$) than that in Ctrl group after weaning.

In conclusion, FML could be used to enhance BW of calves through enhancing apparent digestibility of EE of the diet, and increased the level of serum GH and IGF-1 in calves after the age of 56 days. CT enhanced the BW of calves before weaning through increasing the apparent digestibility of NDF of the diet, and elevated the apparent digestibility of EE of the diet of calves after weaning through increasing the level of serum IGF-1 in calves. Furthermore, the mixture of FML and CT plays a synergistic role in enhancing growth, improving feed intake and nutriment *digestibility*.

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