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Research article

Vitamin D_2 production and *in vitro* ruminal degradation of UV-B irradiated vitamin D enriched yeast in Thai native cattle

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Abstract

The purposes of this study were to investigate the optimum UV-B irradiation time needed to produce vitamin D enriched yeast and to promote *in vitro* ruminal degradation of UV-B irradiated vitamin D enriched yeast in Thai native cattle. *Baker's yeast* and *S. cerevisiae* were irradiated at 0, 2, 4, 8, 10, 12, 16 and 24 hours. The UV-B irradiation of both yeast strains could significantly enhance vitamin D_2 to the highest amount at 16 hours. The corn stover and TMR were studied for determination of *in vitro* ruminal degradation, included three treatments that involved each type of feed, namely feedstuff without supplementation, feedstuff with live yeast supplementation and feedstuff with UV-B irradiated vitamin D enriched yeast. Gas production data of the live yeast group and the UV-B irradiated vitamin D enriched yeast supplementation group, were significantly higher than those of the control groups for both types of feedstuffs. The vitamin D content of the vitamin D enriched yeast supplementation groups were not significantly different from those of the groups subjected to an initial incubation period (10.98 vs. 14.43µg, respectively). Therefore, after a period of irradiation of 16 hours, the two yeasts strain produced the highest vitamin D_2 content, while *Baker's yeast* produced vitamin D_2 at higher amounts than *S. cerevisiae*. Notably, UV-B irradiated vitamin D enriched yeast improved the *in vitro* ruminal degradation, while also preventing a loss in the amount of vitamin D that was degraded by the rumen microorganisms.

Keywords: *Baker's yeast*, *In vitro* ruminal degradation, *Saccharomyces cerevisiae*, UV-B irradiation, Vitamin D_2

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INTRODUCTION

Vitamin D is a fat-soluble vitamin that is naturally present in certain foods. It is essential for life in animals and is involved in the regulation of calcium and phosphate. Vitamin D promotes the absorption of extra-mineral metabolism functions and is needed for the general cellular bioactivities in the body (Rivera et al., 2010). It is primarily obtained endogenously through UV exposure to sun light, while it can also be acquired from dietary supplements and various food sources (Elangovan et al., 2017). Importantly, it must undergo two phases of hydroxylation in the body for activation. The first phase occurs in the liver and converts vitamin D to 25-hydroxyvitamin D (25(OH) D), which is also known as calcidiol. The second phase occurs primarily in the kidneys and forms physiologically active 1, 25-dihydroxyvitamin D (1, 25$(OH)_2$D), which is also known as calcitriol (Dusso and Brown, 1998; Thiangtum et al., 2013). Vitamin D consists of two major compounds that are identified as vitamin D_2 (ergocalciferol) and vitamin D_3 (cholecalciferol). Vitamin D_3 is found in animal products, such as fish liver oil, egg, milk, and cheese, and is mainly generated from 7-dehydrocholesterol in the epidermal layer of the skin as a consequence of exposure to ultraviolet light (Dusso et al., 2005). Vitamin D_2 is found in a number of plant products and is formed through UV radiation from the plant steroid known as ergosterol (Jasinghe and Perera, 2005). Vitamin D_2 is more efficient at bone mineralization than vitamin D_3. In addition, vitamin D_2 does not display a hypercalcemic effect and it is less toxic when compared with vitamin D_3 (Laura et al., 2012). Ergosterol or provitamin D_2 is found in yeast (Fuoli et al., 2008). Importantly, yeast strains are known to be the source of valuable bioactive substances especially ergosterol. These microorganisms play an important role as biotechnological tools used for the production of vitamins and yeasts, while they are also considered high sterol producers. Ergosterol, as well as cholesterol, is present in many animal tissues and skin (where its conversion to vitamin D_2 takes place) (Elena et al., 2013). *Saccharomyces cerevisiae* yeast strains have received a great deal of attention due to its capacity for ergosterol biosynthesis (Reiner et al., 2005; Abe and Hiraki, 2009); furthermore, *S. cerevisiae* yeast is widely used in the production of beer, bread, wine, nutraceuticals, pharmaceuticals and probiotics, there are defined as live micro-organisms which confer a health benefit on the animal (Nielsen and Jewett, 2008; Suwan and Chitmanat, 2017; Piyadeatsoontorn et al., 2018). The resulting products are known to contain a high amount of ergosterol, which can be converted to vitamin D_2. Importantly, when yeast is exposed to UV light, ergosterol undergoes photolysis to yield a variety of photoirradiation products that have principally been identified as previtamin D_2, tachysterol and lumisterol. Previtamin D_2 undergoes a spontaneous thermal rearrangement to be converted to vitamin D_2 (Braun et al., 1991). The conversion of vitamin D_2 by UV light requires the involvement of three sub-regions of wavelengths including UV-C (190-290nm), UV-B (290-320nm) and UV-A (320-400nm) (Teichmann et al., 2007). The highest yield of synthesized vitamin D is dependent upon the absorption of radiation in the ultraviolet-B range (Foss, 2009). Since the duration of the UV-B irradiation was not certain, further studies will be required. However, ultraviolet photoreactions can potentially cause mutations in yeast. Incidences of UV mutagenesis in yeast were not found to have induced gross chromosomal rearrangements (James and Kilbey, 1977), while others indicated an increase in mobile element

Ty transposition (Morawetz and Hagen, 1990). Random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) techniques are able to detect the existence of mutations. In organisms other than yeast, RAPD techniques have been used to distinguish mutations from wild-type specimens (Shafique et al., 2009; 2011). Earlier research studies have determined that the vitamin D supplemented in the diets of ruminants could be degraded in the rumen. Remarkably, only 10 to 25% of added ergocalciferol and cholecalciferol were recovered after 24 hours of incubation in intact ruminal fluid (Sommerfeldt et al., 1979). Since dairy cattle are considered ruminants, the microorganisms in their rumen are not able to convert vitamin D in food to a form that can be utilized (Schmid and Walther, 2013) making it less available to these animals. Nowadays, yeast is used as a feed additive in ruminant feed. Thus, researchers have aimed to increase the activity of rumen microflora by helping to remove oxygen and by increasing the production of volatile fatty acids involved in total short-chain fatty acids (105.4 vs. 116.5 mM), acetate (70.2 vs. 77.3 mM), propionate (24.8 vs. 29.2 mM) and protein microorganism synthesis (292.8 vs. 321.6 g/d). This would ultimately lead to an increase in the production efficiency of these ruminants (Dias et al., 2017). Also, yeast can grow under both aerobic and anaerobic conditions. Yeast typically grows and survives in neutral environments. Even though rumen fermentation can sometimes form an acidic environment (pH 3-4), it can still be resistant to acidic conditions (Walker, 1998) and also reduce ruminal acidosis (Vatsana, 2017). Thus, this research study has aimed to investigate the optimum UV-B irradiation needed time to produce vitamin D enriched yeast and the ruminal degradation of UV-B irradiated vitamin D enriched yeast in Thai native cattle.

MATERIALS and METHODS

Production of UV-B irradiated vitamin D enriched yeast Yeast Yeast strains and their growth conditions

In this study, two types of yeast (*Saccharomyces cerevisiae* and *Baker's yeast*) were cultured. This protocol has been approved (CMUIBC0662003, Approval No. A0662002) by Chiang Mai University Institutional Biosafety Committee. *S. cerevisiae* was obtained from a laboratory at the Department of Biology, Faculty of Sciences, Chiang Mai University. *Baker's yeast* was selected from a single colony of instant active dry yeast using the streak plate method. Two types of yeast culture were plated on YM agar plates (Yeast Malt Medium; Yeast Extract (Difco) 0.3% w/v, Malt extract (Difco) 0.3% w/v, Bactopeptone (Difco) 0.5% w/v, glucose 1% w/v), which were grown at 25 °C for 48 hours. The single randomly selected colony was inoculated on YM broth in sterile test tubes at 25 °C for 24 hours. This yeast culture was then maintained in YM broth containing 25% glycerol at −20 °C on microtubes. The yeast cultures were inoculated from specimens stored at -20 °C on YM broth at 25 °C for 24 hours. The enriched culture (1 % v/v) were used as a starter in 100 mL of YM broth in a 250 mL Erlenmeyer flask and incubated at 25 °C with an agitation rate of 100 rpm. The growth rate optimization of the yeast culture for UV-B irradiation was measured with a spectrophotometer with UV/Visible absorption of the cultured medium samples at 600 nm in 0, 3, 6, 9, 12, 15, 18 and 24 hours. The common growth curve of the microorganisms were composed of the lag, log, stationary and decline phases.

Irradiation procedure

Stock samples of *S. cerevisiae* and *Baker's yeast* were streaked on YM agar plates at 25 °C for 48 hours. A single randomly selected colony was inoculated on YM broth in sterile test tubes at 25 °C for 24 hours. The enriched culture (1 % v/v) was inoculated on YM broth in a sterile Erlenmeyer flask at 25 °C with an agitation rate of 100 rpm for 16 hours. The enriched culture (1 % v/v) was then moved to YM broth and incubated at 25 °C with an agitation rate of 100 rpm for 12 hours. After incubation, specimens of *S. cerevisiae* and *Baker's yeast* were exposed to UV- B irradiation for different periods of duration. Eight units of UV-B lamps (311±5 nm, Philips TL 20W/01 RS SLV/25) at 589.8 mm in length were placed 15 cm away from the sample for irradiation within a total area of 80x120 cm^2. Specimens of *S. cerevisiae* and *Baker's yeast* were treated at 0, 2, 4, 8, 10, 12, 16 and 24 hours in an irradiation chamber. After UV-B irradiation, UV-B irradiated yeast specimens were immediately stored at -20 °C. UV-B irradiated yeast samples were separately freeze-dried and homogenized with a blender before determination and then stored at -20 °C until further analysis.

Analysis of ergosterol and vitamin D_2

UV-B irradiated yeast was extracted and analyzed for ergosterol and vitamin D content according to the established methods of analysis endorsed by AOAC (2000) and Mattila et al. (1994). Approximately 0.5 g of freeze dried UV-B irradiated yeast was weighed and mixed with 1 g L-ascorbic acid in 250 mL round bottom flasks. Subsequently, 50 mL of 95 % ethanol, 10 mL of 50 % potassium hydroxide and 100 µg of cholecalciferol (vitamin D_3; in 1 mL of methanol) were combined to produce an internal standard. The mixture was saponified for 30 min under reflux at 85 °C. The mixture was then cooled down at room temperature and transferred into a separating funnel. The mixture was then twice extracted with 10 mL of deionized water and n-hexane at a volume of 30 mL. The organic layer was washed three times with 50 mL deionized water until it was neutralized. It was then transferred into a round bottom flask, rotary evaporated to dryness at 40 °C and immediately re-dissolved in 1 mL of a mixed solution of eluent (acetonitrile:methanol = 75:25 v/v) and isopropyl alcohol (2:1 v/v). The sample was then filtered through a 0.45 µm non-pyrogenic filter. Five microliters of the filtered sample were injected into an HPLC system (1220 Infinity II LC, Agilent Technologies, USA) and eluted through a reverse phase C18 column (Restek, USA, 5µm, 4.6 x 250 mm). The mobile phase was comprised of acetonitrile:methanol (75:25 v/v) at a flow rate of 1.3 mL/min, while UV detection indicated a volume of 264 nm. Qualitative vitamin D was analyzed by comparing the times of the obtained standards, while quantification was accomplished by using a calibration curve.

Genetic stability of vitamin D enriched yeast

DNA extraction and purification

DNA was extracted from 3 mL YM cultures after 24 hours of UV-B irradiation using a NucleoSpin® Tissue Kit (MACHEREY-NAGEL GmbH & Co.KG, Neanderthal, Germany). Aliquots of the DNA template in amounts of 100 µL were stored at -20 °C. The efficiency of this DNA extraction procedure and the quantity of the extracted DNA were analyzed on electrophoresis 1% (W/V) agarose gel at 100 V for 15 min in 1 x TBE buffer. DNA concentration values were determined with the use of Nano Drop™ 2000/2000c Spectrophotometers.

RAPD-PCR

The protocol established by Legras et al.was employed with slight modifications (Legras et al., 2003). PCR amplification conditions were optimized in a C1000 Touch™ Thermal Cycler (CFX96 Touch Deep Well Real-Time PCR System; BIO-RAD Laboratories, Berkeley, California). A standard RAPD-PCR reaction was performed in a volume of 50 µL containing 25 µL of 2X PCR Taq MasterMix with dye (Applied Biological Materials, Canada). Furthermore, 10 µM of each primer (Pacific science, Thailand), specifically delta12 (5P-TCAACAATGGAATCCCAAC-3P) and delta21 (5P- CATCTTAACACCGTATATGA-3P), were used along with 100 ng of the DNA template and 15 µL of nuclease-free H_2O. The PCR profile was established as follows: 4 min at 94 °C followed by 35 cycles of 30 s at 94 °C, 30 s at 46 °C and 90 s at 72 °C and a finishing step of 10 min at 72 °C. After amplification, PCR products were separated using gel electrophoresis.

Gel electrophoresis

PCR products (5 µL) mixed with 1 µL of 6 x loading buffer (Applied Biological Materials, Canada) were separated by horizontal electrophoresis in 2 % (w/v) agarose gel mixed with RedSafe™ Nucleic Acid Staining Solution (iNtRON Biotechnology, Seongnam, Korea) according to the manufacturer's instructions. DNA molecular mass marker (100 bp+1.5 kb DNA ladder; Life Technologies, Barcelona, Spain) was used as a standard. All electrophoresis procedures were carried out with 15 x 7 cm gels on a Wide Mini-Sub Cell GT unit (15 x 7 cm tray with 13 wells; Bio-Rad Laboratories, Madrid, Spain) at 100 V for 1 hour in 1 x TBE buffer. After electrophoresis, the gel samples were photographed under trans-illuminated UV light using a gel documentation system (BIO RAD; ChemiDocTM MP Imaging System, California, USA).

In vitro ruminal degradation of corn stover and TMR supplemented with UV-B irradiated vitamin D enriched yeast

In vitro gas production techniques

In vitro fermentation was carried out in graduated glass syringes (100 mL capacity) following the method described by Menke et al. (1979). Rumen fluid was collected before the morning feeding from 4 ruminally fistulated Thai native cows (a local breed; BW 250 ± 15 kg) from the Laboratory Animal Center, Chiang Mai University under code of ethics license No. 2563/AG-0002.

The animals were fed corn silage *ad libitum* and 2 kg of concentrate per day. Syringes were filled with 30 mL of medium that consisted of 10 mL of rumen fluid and 20 mL of buffered mineral solution. The experimental diet used in this study was comprised of corn stover (CS) and TMR. The ingredients and chemical compositions of TMR and the corn stover are presented in Table 1. Overall, 3 treatments were prepared for each type of feed as follows;

Treatment 1: without supplementation (control)
Treatment 2: live yeast 0.3g supplementation (Y)
Treatment 3: UV-B irradiated vitamin D enriched yeast 0.3 g supplementation (UV-BY)

Table 1 Ingredients and chemical composition of total mixed rations (TMR).

Item	Ratio (%)	
	Corn stover	TMR
Ingredient		
Rice bran	-	10
Corn meal	-	15
Soybean meal	-	0.5
Sesame meal extracts	-	0.5
Oil palm meal	-	1
Urea	-	1.2
Dicalcium phosphate	-	0.8
Premix minerals	-	0.1
Napier Pak Chong 1 grass	-	60
Corn husk and maize cob	-	10.8
Corn stover	100	-
Chemical composition, DM basis (%)		
Dry matter	23.91	41.77
Organic matter	95.06	91.80
Crude protein	4.87	15.89
Ether extract	3.86	5.66
NDF	61.94	57.53
ADF	41.43	20.84
ADL	5.64	2.97
Hemicellulose	20.51	36.69
Cellulose	52.65	17.87

DM = Dry matter, OM = Organic matter, CP = Crude protein, EE = Ether extract, NDF = Neutral detergent fiber, ADF = Acid detergent fiber, ADL = Acid detergent lignin.

The corn stover and TMR samples were passed through a 1 mm colander until they were ground. Each sample of 230 ± 5 mg was accurately weighed out in 3 replicates and placed into 100 mL glass syringes that were fitted with plungers. They were then incubated in a shaking water bath at 39°C. Three empty syringes containing only the incubation medium were incubated as blanks in order to correct for any gas production resulting from the activity of the rumen fluid. Gas production readings were taken after 12, 24, 48, 72 and 96 hours of incubation according to the moving scale present on the plunger of the glass syringes.

To estimate the fermentation kinetic parameters, data on cumulative gas production were established using the exponential model that had been proposed by Ørskov and McDonald (1979);

$$Y = a + b\,(1-e^{-ct})$$

where
- a = Gas production from the immediate soluble fraction (ml)
- b = Gas production from the immediate insoluble fraction (mL)
- |a|+b = Potential extent of gas production
- c = Gas production rate for the insoluble fraction ($mL.h^{-1}$)
- t = Incubation time (h)
- Y = Gas produced at time't'

Metabolizable energy (ME, MJ/kg DM) and) NEL were estimated using CP and EE contents (in g/kg DM) and the volume of gas production was measured after 24 hours of incubation (G24 in mL per 200 mg DM incubated) as has been described by Menke and Steingass (1988);

$$ME_{corn}\ (MJ/kg\ DM) = 2.20 + 0.1357GP + 0.0057CP + 0.0002859(CA)^2 \quad (R=0.94)$$
$$ME_{TMR}\ (MJ/kg\ DM) = 0.72 + 0.1559GP + 0.0068CP + 0.0249EE \quad (R=0.95)$$
$$NEL_{corn}\ (MJ/kg\ DM) = 0.54 + 0.0959GP + 0.0038CP + 0.0001733(CP)^2 \quad (R=0.93)$$
$$NEL_{TMR}\ (MJ/kg\ DM) = -0.61 + 0.1138GP + 0.0046CP + 0.0150EE \quad (R=0.95)$$

where
- GP = 24 hours net gas production (ml/200 mg DM)
- CP = Crude protein (%DM)
- EE = Ether extract (%DM)
- CA = Crude ash (%DM)

The syringes were categorized into separate sets of sample incubations for the determination of gas production kinetics. The parameters were calculated for *in vitro* dry matter digestibility (IVDMD) and *in vitro* organic matter digestibility (IVOMD). Approximately 500 mg of the samples were weighed and incubated following the *in vitro* gas production techniques that had been previously described. After an incubation period of 24 hours, the contents of the syringes were transferred into a Buchner funnel and then the residue was filtered. The water was removed by baking the residue at 100 °C for apparent degradable substrate calculation;

$$IVDMD\ (\%) = \frac{(DM\ Initial\ weigh - DM\ final\ weigh)}{DM\ Initial\ weigh}\ x\ 100$$
$$IVOMD\ (\%) = \frac{(OM\ Initial\ weigh - OM\ final\ weigh)}{OM\ Initial\ weigh}\ x\ 100$$

The microbial biomass yield (MBY) procedures that were followed were similar to those described by Blümmel et al. (1997). Approximately 500 mg of the samples were weighed and incubated by following the *in vitro* digestibility method that had been described previously. The incubation process was terminated after 24 hours, the entire contents of the syringes were transferred into beakers for true degraded substrate determination. Whole contents were digested with neutral detergent soluble (NDS) according to the neutral detergent fiber (NDF) method. The filtered residue was then dried and incinerated to determine the dry matter content and organic matter, respectively.

The MBY was modified in order to be expressed as mg/ 100mg of the truly digested sample according to the method described by Blümmel et al. (1997);

$$\text{MBY} = \frac{(\text{truly digested sample} - \text{apparently digested sample})}{\text{truly digested sample}}$$

***In vitro* ruminal degradation of vitamin D and volatile fatty acid production**

The degradation of UV-B irradiated vitamin D enriched yeast was achieved according to the method of Menke et al. (1979). For the *in vitro* ruminal degradation of vitamin D, 230±5 mg of corn stover and TMR following the treatment described above were mixed with 30 mL of rumen liquor buffer solution into a 125 mL serum bottle, and then closed with a rubber stopper and an aluminum cap. After that, the cap was clamped tightly shut with a crimper hand seal ring. Except for the corn stover and TMR samples, three replicates of the bottles containing commercial vitamin D and rumen liquor buffer solution were used for standard vitamin D ruminal degradation analysis. Samples were incubated at 39 °C with an agitation rate of 70 rpm for 24 hours and then stored at -20 °C in order to analyze the vitamin D content following the same method described above. For *in vitro* ruminal VFA's production, serum bottles containing corn stover and TMR prepared by the treatment described above were categorized into separate groups of samples according to different incubation periods for the determination of volatile fatty acid (VFA) contents by high performance liquid chromatography (HPLC). The total contents of the serum bottles were transferred into centrifuge tubes. The residue samples were then centrifuged in an ultracentrifuge at 10,000 g for 10 min at 4 °C. The supernatant fraction was carefully filtered through a 0.45 µm non pyrogenic filter into a tapered vial prior to analysis. Twenty microliters of the filtered samples were injected into an HPLC system (E2695 Aminex®, Alliance Waters, USA) and eluted through HPX-87H Ion Exclusion Column (Bio-Rad, USA, 9 µm, 7.8 × 300 mm). The mobile phase was comprised of 0.02 N sulfuric acid (H_2SO_4) at a flow rate of 1 mL/min and UV detection was recorded at 210 nm. The qualitative content of vitamin D was analyzed by comparing the times of the obtained standards and quantification was established by using a calibration curve.

Statistical analyses

All data were analyzed using the R program (Version R-4.0.4). Analysis of variance (ANOVA) for complete randomized design (CRD) was employed to analyze any differences in the mean values within each group. The Shapiro-Wilk test and Levene's test were used to evaluate the normality and homogeneity of variance assumptions. Mean differences among treatments were determined using Duncan's new multiple range test (Steel and Torrie, 1980).

RESULTS

The optimization of UV-B irradiation was determined by growth rate. Subsequently, specimens of *S. cerevisiae* and *Baker's yeast* were optimized under conditions involving 12 hours of incubation at 25 °C with an agitation rate of 100 rpm. Specifically, 0-6 hours of the incubation period were categorized as the lag phase. In the early stage, yeast specimens were allowed to adapt to the new environment, while the amount of yeast did not increase. During 6-12 hours of the incubation period, yeast specimens rapidly and steadily underwent cell division, for which growth rates were found to be the highest. This period was known as the log phase or the exponential phase. After 12 hours, yeast cell division took place as the specimens entered the stationary phase. At this point, yeast specimens were present in the highest amounts and no increase in cell division was observed. Consequently, the incremental rate was equal to the mortality rate as nutrients were almost completely used up, as is shown in Figure 1.

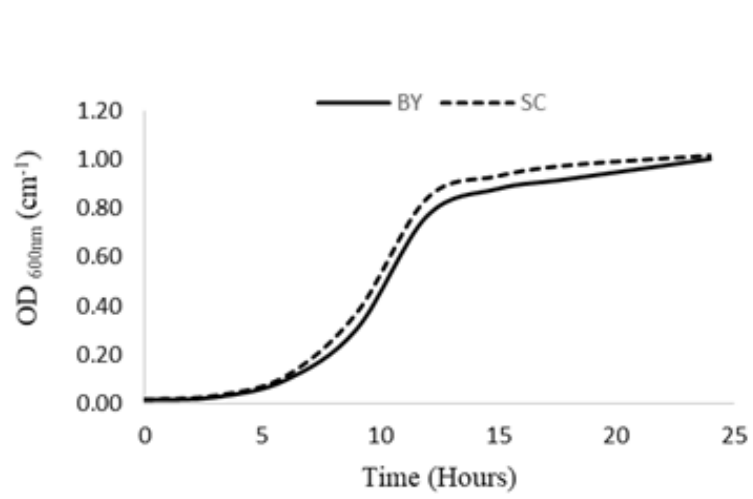

Figure 1 Growth rate of *S. cerevisiae* (SC) and *Baker's yeast* (BY) in YM broth cultures.

The vitamin D_2 contents of the yeast specimens after UV-B irradiation at different periods were varied. Vitamin D_2 concentrations of *S. cerevisiae* and *Baker's yeast* in the control (unirradiated) were 1,569.87±37 and 814.49±58 IU/100g DM, respectively. The amount of vitamin D_2 from the *Baker's yeast* specimen was the highest after UV-B irradiation for 16 hours when compared with the initial period of irradiation (814.49±58 IU/100g DM vs. 3,623,174.08±7,331 IU/100g DM). In the same way, *S. cerevisiae* increased to the highest amount of vitamin D2 from 1,569.87±37 IU/100g DM (unirradiated) to 2,437,194.38±5,508 IU/100g DM after UV-B irradiation for 16 hours, which was significantly higher than for the UV-B irradiation periods of 0, 2, 4, 8 and 10 hours ($P < 0.05$). UV-B irradiation at different periods were increased at unequal rates for each yeast strain. The *Baker's yeast* specimens revealed significantly increased amounts of vitamin D_2 that were higher than *S. cerevisiae* ($P < 0.05$) as is shown in Figure 2 (A). After 16 hours of UV-B irradiation, the amount of vitamin D_2 began to significantly decrease ($P < 0.05$).

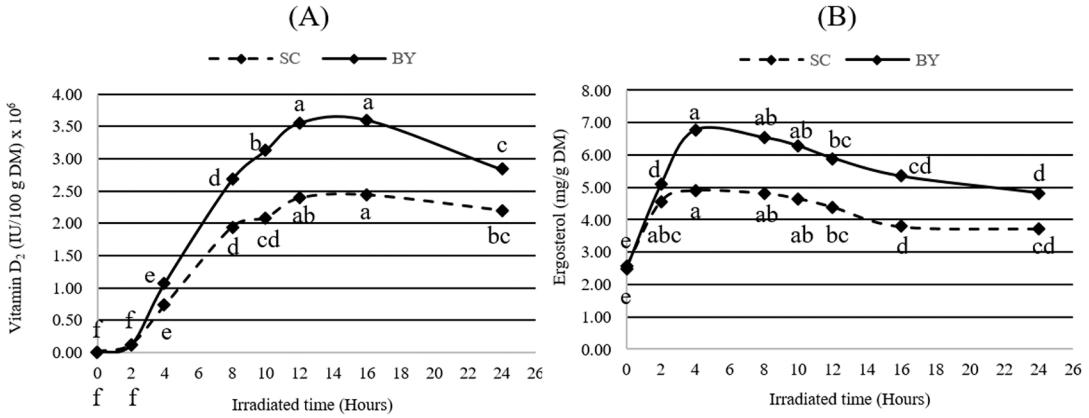

Figure 2 Effect of UV-B irradiation time on vitamin D_2 and ergosterol content of *S. cerevisiae* (SC) and *Baker's yeast* (BY). The $^{a, b, c, d, e, f}$ mean values along columns among irradiated times with different superscripts were determined to be significantly different at $P < 0.05$.

The ergosterol contents of *S. cerevisiae* and *Baker's yeast* increased after UV-B irradiation. After 4 hours of UV-B irradiation, the ergosterol content of the two yeast strains decreased. The ergosterol content of the *Baker's yeast* strain was significantly higher than that of the *S. cerevisiae* strain ($P < 0.05$), as is shown in Figure 2B. Therefore, an irradiation time of 12 hours for the *Baker's yeast* was selected to be tested by the *in vitro* gas production technique and ruminal degradation of UV-B irradiated vitamin D enriched yeast was then determined in the rumen.

The genetic stability of UV-B irradiated vitamin D enriched yeast was analyzed. The genetic profiles of the *S. cerevisiae* strain are showed on numbers 8, 9 and 10 and compared with 11, 12 and 13, respectively. Similarly, the profiles of the Baker's yeast strain before and after UV-B irradiation are shown on numbers 2, 3 and 4 and compared with 5, 6 and 7, respectively. The location of the band on the gel electrophoresis of Baker's yeast and *S. cerevisiae* strains were compared with the values 2:5, 3:6, 4:7 and 8:11, 9:12, 10:13, respectively. In this case, no differences were observed in specimens with or without UV-B irradiation for values obtained with primer delta12 and delta 21, as is shown in Figure 3. The genetic profile of each strain was analyzed after UV-B irradiation demonstrated an identical genetic profile to that of the microbank control strain reported by RAPD-PCR. Consequently, UV-B irradiation had no effect on mutations in the *S. cerevisiae* and *Baker's yeast* strains.

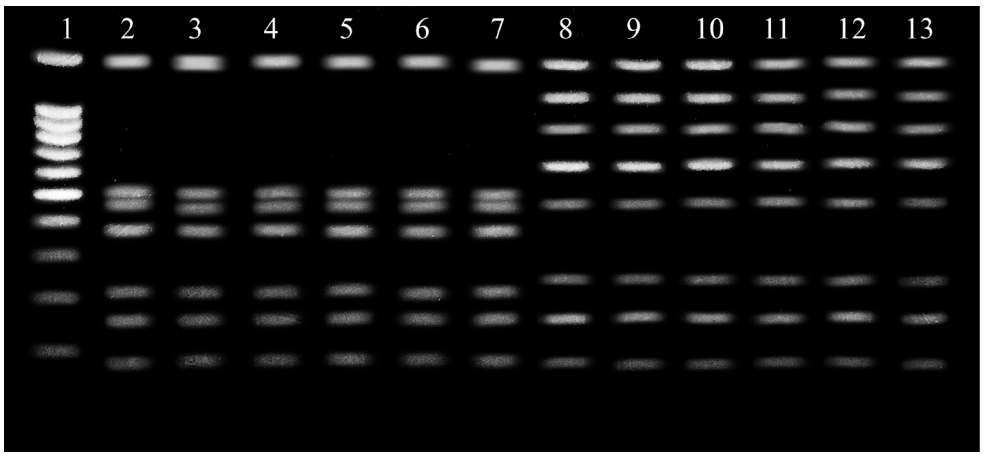

Figure 3 Representative RAPD-PCR fingerprints of *S. cerevisies* and *Baker's yeast* with or without UV-B irradiation obtained with primer delta12 and delta 21.

Gas production at 12, 24, 48, 72 and 96 hours of the CS+Y (48.38, 64.01, 79.23, 86.84 and 90.65 mL/0.2 g DM), CS+UV-BY (50.41, 65.76, 81.03, 88.97 and 92.16 mL/0.2 g DM) groups were significantly higher than those of the CS (15.23, 22.53,33.52, 38.23 and 44.43 mL/0.2 g DM) groups, while the TMR+Y (59.02, 75.39, 90.42, 96.46 and 97.94 mL/0.2 g DM), TMR+UV-BY (59.72, 78.61,93.34, 98.05 and 100.87 mL/0.2 g DM) groups were significantly higher than those of the TMR (27.61, 43.30, 53.60, 57.35 and 61.09 mL/0.2 g DM) group ($P < 0.05$), respectively. Similarly, the CS+Y, CS+UV-BY and TMR+Y, and TMR+UV-BY groups revealed gas production values from the degradable fraction (b), the potential extent of gas production (|a|+b), the gas production rate (c), metabolizable energy (ME), Net energy for lactation (NEL), acetic acid, propionic acid, butyric acid and microbial biomass yield (MBY; mg/500mgDM, % of true digestible) that were significantly higher than those of the CS and TMR groups ($P < 0.05$), respectively. Furthermore, the gas production values from the soluble fraction (a), *in vitro* dry matter digestibility (IVDMD) and *in vitro* organic matter digestibility (IVOMD) of the CS+Y, CS+UV-BY and TMR+Y, and TMR+UV-BY groups were significantly lower than those of the CS and TMR groups ($P < 0.05$), respectively (Table 2).

Table 2 *In vitro* gas production and gas kinetics of corn stover and TMR with or without yeast, and vitamin D enriched, yeast at different periods of incubation.

Items	Corn			SEM	P-value	TMR			SEM	P-value
	Control	Yeast	UV-B Yeast			Control	Yeast	UV-B Yeast		
***In vitro* gas production (mL/0.2 g DM)**										
12 hours	15.23^{A}	48.38^{B}	50.41^{B}	5.71	**	27.61^{X}	59.02^{Y}	59.72^{Y}	5.20	**
24 hours	22.53^{A}	64.01^{B}	65.76^{B}	7.24	**	43.30^{X}	75.39^{Y}	$78..61^{Y}$	5.54	**
48 hours	33.52^{A}	79.23^{B}	81.03^{B}	8.01	**	53.60^{X}	90.42^{Y}	93.34^{Y}	6.30	**
72 hours	38.23^{A}	86.84^{B}	88.97^{B}	8.56	**	57.35^{X}	96.46^{Y}	98.05^{Y}	6.52	**
96 hours	44.43^{A}	90.65^{B}	92.16^{B}	8.79	**	61.09^{X}	97.94^{Y}	100.87^{Y}	6.30	**
Kinetics of gas production										
a (mL.)	1.18^{C}	-1.41^{A}	-0.06^{B}	0.49	**	-3.29^{X}	-6.83^{Z}	-5.42^{Y}	0.54	**
b (mL./0.2 g DM)	49.29^{A}	89.85^{B}	88.66^{B}	8.53	**	64.03^{X}	104.28^{Y}	104.35^{Y}	6.62	**
c (% / hr.)	0.032^{A}	0.058^{B}	0.065^{C}	0.01	**	0.050^{X}	0.063^{Y}	0.070^{Z}	0.01	**
|a|+b	50.47^{A}	91.46^{B}	89.01^{B}	8.41	**	67.31^{X}	111.11^{Y}	109.76^{Y}	7.09	**
Estimated parameters										
ME (Mj/Kg DM)	5.29^{A}	10.93^{B}	11.17^{B}	0.98	**	7.72^{X}	12.72^{Y}	13.23^{Y}	0.86	**
NEL (Mj/Kg DM)	3.88^{A}	4.04^{B}	4.05^{B}	0.03	**	3.54^{X}	3.69^{Y}	3.70^{Y}	0.04	**
Ruminal VFA's (mmol)										
Acetic acid	11.96^{A}	24.39^{B}	25.90^{C}	2.21	**	13.55^{X}	25.37^{Y}	27.73^{Y}	2.23	**
Propionic acid	5.68^{A}	10.43^{B}	10.61^{B}	0.81	**	7.87^{X}	11.73^{Y}	12.49^{Y}	0.76	**
Butyric acid	2.18^{A}	5.39^{B}	5.55^{B}	0.55	**	2.31^{X}	5.48^{Y}	5.83^{Y}	0.54	**
Digestibility (%)										
IVDMD	31.55^{B}	24.26^{A}	25.28^{A}	1.55	**	39.68^{Y}	33.61^{X}	35.74^{XY}	1.07	**
IVOMD	33.18^{B}	25.52^{A}	26.59^{A}	1.63	**	43.22^{Y}	36.61^{X}	38.93^{XY}	1.17	**
True digestibility	37.70	41.65	42.29	1.02	N.S.	51.72^{X}	55.17^{Y}	56.95^{Y}	0.97	**
MBY										
(mg/500mgDM)	30.85^{A}	86.99^{B}	85.04^{B}	11.66	**	60.24^{X}	107.80^{Y}	106.10^{Y}	7.90	**
(% of true digestible)	17.83^{A}	45.80^{B}	44.04^{B}	5.77	**	25.23^{X}	42.19^{Y}	40.30^{Y}	2.74	**

$^{A, B, C}$ mean values along columns among treatment groups in corn stover with different superscripts were determined to be significantly different at $P < 0.05$, $^{X, Y, Z}$ mean values along columns among treatment groups in TMR with different superscripts were determined to be significantly different at $P < 0.05$, SEM = standard error of mean, ** = significantly different at $P < 0.05$, N.S = non significantly different at $P > 0.05$, a = gas production from the soluble fraction, b = gas production from the degradable fraction, c = gas production rate, |a|+b = potential extent of gas production, ME = Metabolizable energy, NEL = Net energy for lactation, IVDMD= *In vitro* dry matter digestibility, IVOMD = *In vitro* organic matter digestibility, MBY = Microbial biomass yield.

The amount of vitamin D in the corn stover and TMR specimens were unable to be measured (non-detected) in the control and yeast supplementation groups. After 24 hours of incubation, the vitamin D content of the vitamin D enriched yeast supplementation group for the corn stover and TMR specimens decreased when compared with the initial supplementation procedure (231.45µg to 220.47µg and 217.02µg or remained about 95.25% and 93.76%, respectively). In order to evaluate the reduction in vitamin D content among the treatment groups, it was found that the degree of reduction in the vitamin D enriched yeast supplementation groups were not significantly different from those of the initial supplementation group (10.98µg vs. 14.43µg or 4.74 % vs 6.23%, respectively) ($P > 0.05$). Contrastively, the amounts of vitamin D reduction in the vitamin D_2 and vitamin D_3 groups were higher than in the CS+UV-BY and TMR+UV-BY groups (115.40µg and 120.42µg vs. 10.98µg and 14.43µg or 50.07% and 54.64% vs. 4.74% and 6.23%, respectively) ($p<0.05$). As is shown in Table 3, the CS+UV-BY and TMR+UV-BY groups revealed higher remaining vitamin D content after 24 hours of incubation than for the vitamin D_2 and vitamin D_3 groups (220.47µg and 217.02µg vs.115.07µg and 99.92µg or 95.25% and 93.76% vs. 49.93% and 45.36%, respectively) ($P < 0.05$), as is shown in Table 3.

Table 3 Vitamin D content and degradation after 24 hours of incubation in rumen liquid buffer *in vitro* with or without yeast culture and UV-B irradiated

Item	Vitamin D commercial		Corn stover			TMR			SEM	P-value
	Vitamin D_2	Vitamin D_3	Control	Yeast	UV-B yeast	Control	Yeast	UV-B yeast		
Initial of vitamin D (µg)	230.47±1.75	220.34±1.26	N.D.	N.D.	231.45±5.01	N.D.	N.D.	231.45±5.01	-	-
Remaining of vitamin D										
(µg)	115.07^{A}±3.6	99.92^{A}±13.5	N.D.	N.D.	220.47^{B}±0.9	N.D.	N.D.	217.02^{B}±10.2	3.14	**
(%)	49.93^{A}±1.1	45.36^{A}±3.4	N.D.	N.D.	95.25^{B}±0.4	N.D.	N.D.	93.76^{B}±4.4	1.35	**
Disappearance of vitamin D										
(µg)	115.40^{B}±5.3	120.42^{B}±13.9	N.D.	N.D.	10.98^{A}±0.9	N.D.	N.D.	14.43^{A}±10.2	3.14	**
(%)	50.07^{B}±1.5	54.64^{B}±3.4	N.D.	N.D.	4.74^{A}±0.4	N.D.	N.D.	6.23^{A}±4.4	1.35	**

$^{A, B}$ mean values along columns among treatment groups with different superscripts were determined to be significantly different at $P < 0.05$, ** = significantly different at $P < 0.05$, SEM: standard error of mean, ND: not detected.

DISCUSSION

At the early stage, the vitamin D_2 content of the yeast strains increased after UV-B irradiation because a high degree of accumulation of ergosterol or provitamin D_2 was present within the yeast cells. When stimulated by UV-B light, vitamin D could be converted into vitamin D_2 (Elena et al., 2013). The specimens contained a high amount of ergosterol that could be converted into vitamin D_2. When the yeast strains were exposed to UV light, ergosterol underwent photolysis to yield a variety of photoirradiation products, principally previtamin D_2, tachysterol and lumisterol. Consequently, previtamin D_2 underwent spontaneous thermal rearrangement to vitamin D_2 (Braun et al.,1991). After 16 hours of UV-B irradiation, the amount of vitamin D began to significantly decrease ($P < 0.05$). Due to the fact that the yeast cells began to die and self-decompose (autolysis), the enzymes inside the yeast cells digested various substrates that led to the thinning of the cell wall and the loss of semi-permeable membrane properties. Remarkably, yeast cells decompose themselves until they reach cell lysis to yield intracellular fluids comprised of cytoplasm, fat, protein and vitamins. This fluid was subsequently released outside of the cells (Arnold, 1972). *Baker's yeast* strains revealed significantly increased amounts of vitamin D_2 that were higher than for *S. cerevisiae* because *Baker's yeast* contained more ergosterol than *S. cerevisiae*, as is illustrated in Figure 2B. The genetic profile of each strain with or without UV-B irradiation had no effect on the genetics of the two yeast strains, which were reported from the same position of each band on gel electrophoresis.

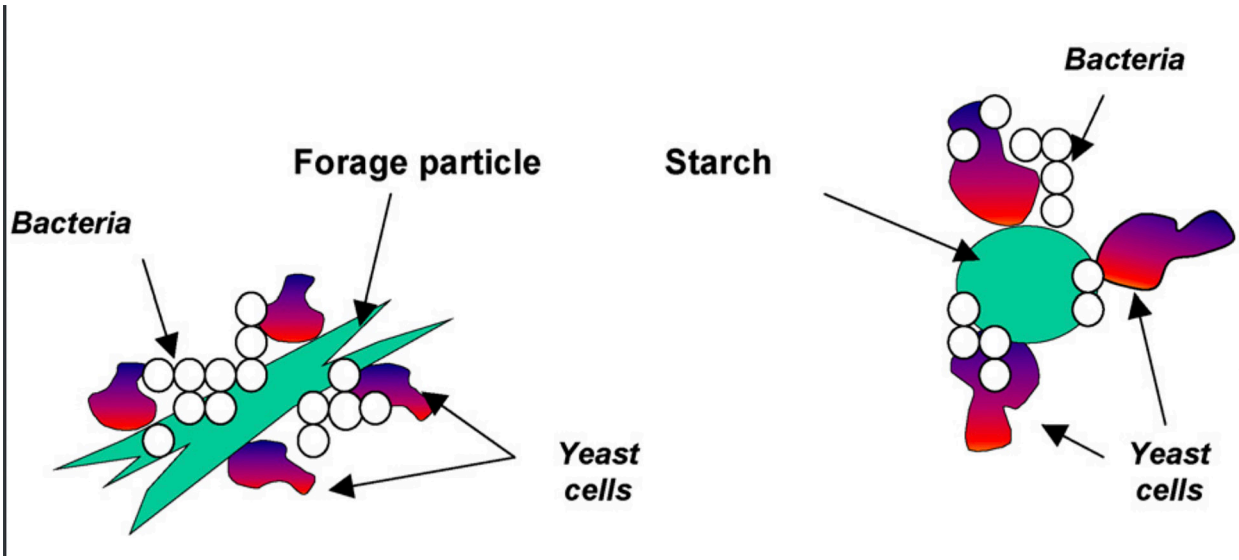

Figure 4 Schematic depiction of yeast cells using oxygen located within and immediately around freshly ingested solid particles. Source: Jouany (2006)

Gas production values of the yeast strain group and the vitamin D enriched yeast supplementation group were higher than for those in the group without yeast supplementation. It is believed that this occurred because yeast can stimulate the growth and activity of fibrolytic bacteria. Furthermore, yeast can compete for the glucose and oligosaccharide obtained through the digestion of amylolytic bacteria that were attached to the feedstuffs in order to provide low amounts of glucose for bacteria utilization (*Streptococcus bovis*) (Jouany et al., 1999), as is shown in Figure 4. Girard and Dawson (1995) reported that yeast could induce the growth of *F. succinogenes S85* and reduce the resting stage of *R. albus7*, *R. flavefaciens FD1* and *Butyrivibrio fibrisolvens D7*. Yeast was considered a growth factor for microorganisms in the rumen. Yeast can also utilize oxygen that may have come in with the feedstuff or water that the animals may have ingested. Yeast uses oxygen for the metabolism of the yeast itself as rumen is anaerobic. This can be considered ideal conditions for the growth of anaerobic microorganisms within the rumen, especially for those of fibrolytic bacteria (Fonty and Chaucheyras-Durand, 2006) and the microorganisms increase markedly to response on fiber digestion (Kovitvadhi et al., 2019) as is illustrated in Figure 5. Furthermore, yeast was determined to be a potent source of probiotics in ruminants that can stimulate the growth of microbes in the rumen (Jouany et al., 2006).

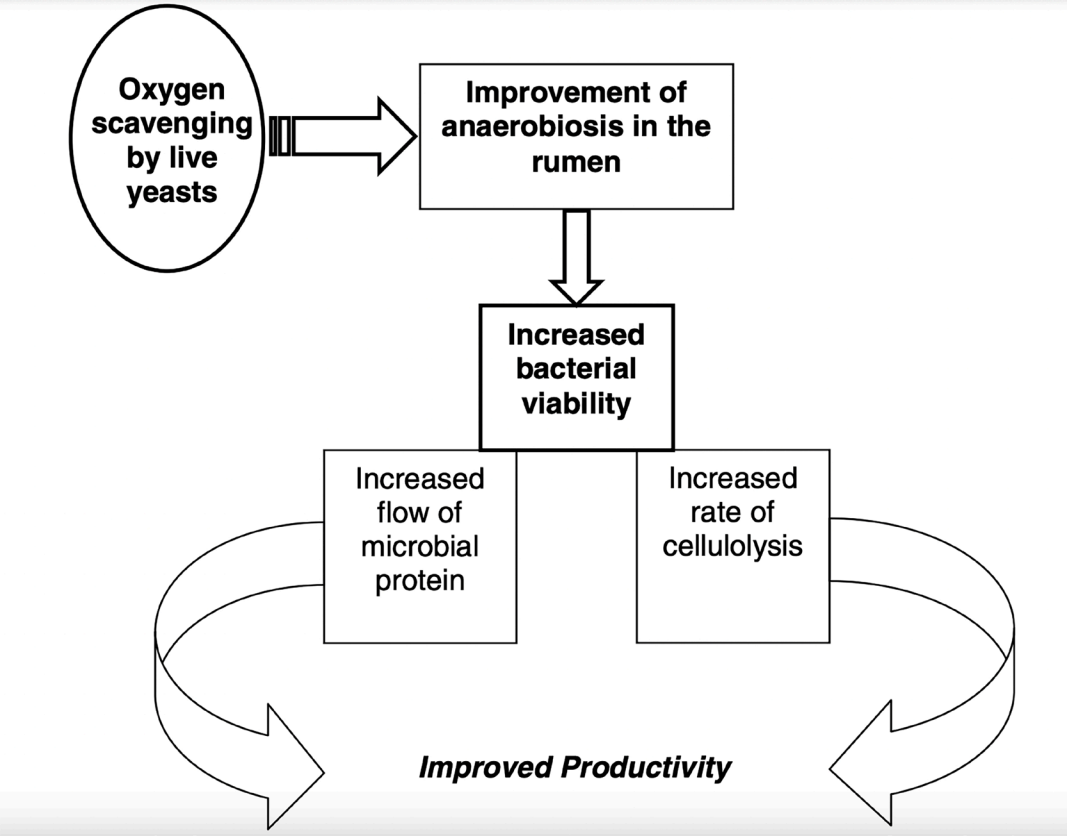

Figure 5 Importance of oxygen scavenging by live yeast strains in terms of driving bacterial viability in rumen and its consequences on animal production. Source: Fonty and Chaucheyras-Durand (2006)

As a result, yeast culture supplementation may promote the increased digestibility of fibers (Chaucheyras-Durand and Fonty, 2001; Ettle and Schwarz, 2002). Similarly, Kim et al. (1992) reported that yeast culture supplementation could increase the digestibility of CF, NDF and ADF. Thus, it could then increase total VFA's from a high degree of fiber digestibility in the rumen (Galip et al., 2006). Accordingly, with regard to the kinetics of gas production, the ME, NEL and VFA's of the yeast and vitamin D enriched yeast supplementation groups were higher than for those of the group without yeast supplementation because yeast can improve digestibility in the rumen. Normally, the rumen contains microorganisms that degrade or convert vitamin D into other substances that animals cannot utilize. As a result, the direct supplementation of vitamin D supplementation in dairy cow diets could be degraded by ruminal microorganisms. In accordance with Sommerfeldt et al. (1980), a study on the degradation of vitamin D in the rumen revealed that the experimental group that was supplemented with vitamin D in the non-sterilized rumen liquor buffer displayed a decrease in vitamin D content by 50–75% when compared to the sterilized rumen liquor buffer group. In the vitamin D enriched yeast supplementation group, in both corn stover and TMR trials, slightly reduced vitamin D contents were revealed after 24 hours of incubation (4.74% and 6.23%, respectively). This was because yeast can grow under both aerobic and anaerobic conditions. Even though rumen fermentation can sometimes be formed in acidic environments (pH 3-4), it was found to be resistant to acidic conditions (Walker, 1998). Yeast can utilize glucose and oligosaccharide produced by the digestion of amylolytic bacteria in the production of energy for their growth. Importantly, they can survive in the rumen without being degraded by microbes that are present in the rumen (Jouany et al., 1999; 2006). Therefore, vitamin D in yeast cells is not degraded by rumen microorganisms.

CONCLUSIONS

Both *S. cerevisiae* and *Barker's yeast* could produce the highest amounts of vitamin D_2 after an irradiation time of 16 hours. *Barker's yeast* could produce higher vitamin D_2 contents than *S. cerevisiae*. The UV-B irradiation had no effect on mutation in the Baker's yeast and *S. cerevisiae* strains. Notably, vitamin D enriched yeast supplementation in TMR could improve digestibility better than in the group supplemented with corn stover. However, vitamin D enriched yeast could prevent the loss of vitamin D that is typically degraded by rumen microorganisms. This may enhance vitamin D to be delivered directly to the small intestines, allowing animals to absorb vitamin D that can then be fully utilized. However, full confirmation of this outcome would still require further studies on UV-B irradiated vitamin D enriched yeast in the diets of dairy cows. These further studies would allow researchers to determine which animals could better utilize the vitamin D that is bypassed from the rumen.

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AUTHOR CONTRIBUTIONS

Patipan Hnokaew: contributed to the design and implementation of the research, to the analysis of the results and to the writing of the manuscript.
Saowaluck Yammuen-Art: contributed to the design and implementation of the research, to the analysis of the results and to the writing of the manuscript.

CONFLICT of INTERESTS

The authors declare that they have no competing interests.

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