Effect of pineapple stem starch feeding on rumen microbial fermentation, blood lipid profile and growth performance of fattening cattle

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ABSTRACT

Pineapple stem starch (PS) was evaluated for its suitability as a new starch source in concentrate for fattening cattle, based on the growth performance, blood profile and rumen parameters of 36 steers in a 206-day feeding study. PS was formulated as a 40% concentrate and fed with forage in comparison with ground corn (GC) and ground cassava (CA) formulated at the same level. PS feeding improved weight gain and feed conversion ratio without affecting feed intake. PS did not obviously influence blood lipid profiles throughout the experiment. Ruminal concentration of total short chain fatty acids (SCFA) increased with PS without affecting SCFA composition throughout the feeding study. Rumen amylolytic group, especially *Ruminococcus bromii*, was dominant in the rumen microbial community, and showed increased abundance by PS feeding throughout the experiment. These results clearly indicate the potential of PS as a useful starch source for fattening cattle in terms of rumen fermentation and growth performance.

Key words: *fattening cattle, growth, pineapple stem, rumen bacteria, starch*

1. INTRODUCTION

Corn and cassava are readily available and are the most popular starch sources in Thailand and other Southeast Asian countries. However, the cost of these starch sources has risen due to increasing interest in their use for animal feed, especially in ruminant livestock. Indeed, corn and cassava as ruminant feed ingredients have become highly competitive (Wanapat *et al.* 2012). On the other hand, byproducts of agricultural and pharmaceutical industries have replaced some corn and cassava recently. One such byproduct is pineapple stem starch (PS), the residue from the extraction of bromelain enzyme, which is mainly intended for pharmaceutical use. PS is high in starch content, in particular amylose, compared with corn and cassava (Nakthong *et al.* 2017). The availability of PS in terms of amount and price may allow this material to be applied to animal feed as an alternative cost effective energy source to corn and cassava. However, no detailed evaluation in relation to demand and impact on the feed market has been made for potential application of PS to ruminant livestock, in particular fattening cattle, the main target of such new starch sources.

Ruminal hydrolysis of a starchy diet is not simply a process by which α−1, 4 and 1, 6 linkages are cleaved by amylase enzymes, as other factors besides starch breakdown exist. In fact, among the many starch sources available as a concentrate ingredient, considerable variations exist in chemical and physical characteristics including proportions of amylose and amylopectin, starch-associated lipids and proteins, and size of starch granules (Svihus *et al.* 2005;

Stevnebo *et al.* 2009), all of which affect the rate and extent of starch hydrolysis and fermentation in the rumen. Therefore, all of these factors need to be considered, especially when a new starch source is evaluated.

One of the most important measures in the evaluation of starch sources is the response of rumen microbes and associated fermentation products. The amylolytic activity of rumen bacteria increases with starch availability (Noziere & Michalet−Doreau 1997). The rumen amylolytic group is mainly composed of *Ruminococcaceae* (Xia *et al.* 2015) including *Ruminococcus bromii*, *Prevotellaceae* represented by *Prevotella ruminicola*, and the genus *Butyrivibrio* (Wallace *et al.* 1997). Of these, *R. bromii* is of interest for its superior hydrolysis and fermentation of starch (Mukhopadhya *et al.* 2018) as well as the involvement of amylases (Jung *et al.* 2019). This species is considered to be an ecological indicator of efficient and safe starch utilization in the rumen of fattening cattle on a high grain diet (Koike *et al.* unpublished results). Similarly, *R. bromii* is known to be a dominant bacterial species in the rumen of cattle fed a barley-based diet (Klieve *et al.* 2007). Thus, it is of interest to determine ruminal responses to the new candidate starch source PS under a practical feeding condition of fattening cattle in areas where PS is available.

The present study aimed to evaluate the applicability of PS as a feed ingredient for fattening cattle through tracking growth performance, blood lipid profile and rumen microbial responses to a dietary formulation of PS.

2. MATERIALS AND METHODS

97 The experimental protocol was reviewed and approved by the Animal Usage
98 and Ethics Committee of Kasetsart University, Thailand (ACKU62-AGK-007).

99

100 **2.1 Animals, experimental design and feeding**

101 The experiment was conducted at the Ruminant Research Unit, Department of
102 Animal Science, Faculty of Agriculture at Kamphaeng Saen, Kasetsart University,
103 Nakhon Pathom, Thailand. Thirty-six Holstein steers with an average body weight
104 of 453 ± 35.3 kg at 22 months of age were managed in open-air pens (11.25 m^2 for
105 two steers per pen) with rubber mats on concrete floors. The steers were divided
106 into three groups of twelve steers each, according to body weight. Steers were
107 allotted to one of three dietary treatments including a concentrate of corn, cassava
108 or pineapple stem starch. Steers were fed the concentrate *ad libitum* with Napier
109 grass silage (3 kg/head/day in DM) from day 1 to 74. From day 75 to 206, steers
110 were fed the same concentrate with Napier grass silage (2 kg/head/day in DM) and
111 rice straw (0.9 kg/head/day in DM) as roughage. The experimental diet
112 (concentrate and forage) was offered twice a day at 07:00 and 16:00 h

113 The three different starch sources (Table 1) in concentrate were: i) ground corn
114 (GC), ii) ground cassava (CA), and iii) pineapple stem starch (PS), and these were
115 formulated as a 40% concentrate so that the starch content was equalized (Table
116 2). Ground corn and ground cassava were purchased from Cowboyfriend Co., Ltd.,
117 Nakhon Pathom, Thailand, while pineapple stem starch was obtained from Hong
118 Mao Biochemicals Co., Ltd., Rayong, Thailand. Napier grass silage was prepared
119 at the farm of Kasetsart University. The chopped length of the silage was

120 approximately 2−3 cm. Rice straw was purchased from a dealer in Suphanburi
121 province, Thailand.
122 Dry matter intake (DMI) was recorded daily by weighing the offered and
123 refused amounts. Body weight was measured using a walk-through scale before
124 morning feeding once monthly during the experiment. Average daily gain (ADG)
125 and feed conversion ratio (FCR) were calculated to evaluate growth performance.
126
127 **2.2 Feed sampling and analyses**
128 Starch sources and feeds were sampled every two weeks. Silage was assayed
129 for DM (AOAC 2016) in order to adjust the amount fed. All samples were frozen
130 (−20 °C) and mixed to obtain a representative sample for the analysis of crude
131 protein, ether extract, neutral detergent fiber (NDF), acid detergent fiber (ADF),
132 crude ash (AOAC 2016) and starch (ISO 2000).
133 The starch granule size distribution of the three starch sources was analyzed
134 using an image analyzer (KS400 v2; Carl Zeiss, Cambridge, UK) connected to a
135 Carl Zeiss Axiophot 2 microscope. Before analysis, starch samples were mixed with
136 an 80% sucrose solution following the method described by Baldwin (1994).
137
138 **2.3 Blood sampling and analyses**
139 Blood samples (5 mL) were obtained from steers at 4 h after morning feeding
140 on days 74, 147 and 206 of the experiment by puncture of the jugular vein of each
141 steer. Blood was collected in a heparinized glass tube. Plasma was separated by
142 centrifugation for 15 min (3,000 × *g*) at 4 °C. The plasma was used for analysis of
143 low-density lipoprotein (LDL) with a MULTIGENT direct LDL reagent kit (Abbott,

144 Abbott Park, IL, USA. High-density lipoprotein (HDL) was also measured with an
145 Ultra HDL reagent kit (Abbott). Triglycerides and cholesterol were analyzed using
146 a triglyceride reagent kit and a cholesterol reagent kit, respectively (Abbott). Very
147 low-density lipoprotein (VLDL) was calculated according to the equation: VLDL =
148 triglycerides/5 (Friedewald *et al.* 1972).
149
150 **2.4 Rumen sampling and analyses**
151 Rumen fluid (approximately 50 mL) was collected by using a manual vacuum
152 pump connected to a stomach tube at 4 h after the morning feeding on days 74, 147
153 and 206 of the experiment. The collected samples were immediately strained
154 through two layers of cheesecloth. Ruminal pH was immediately measured (pH
155 meter probe, Oakton WD 35634-30, Vernon Hills, IL, USA). These samples were
156 frozen to determine ammonia nitrogen (NH_3–N), short chain fatty acids (SCFA),
157 lactate (−20 °C) and microbes (−80 °C). NH_3–N was spectrophotometrically
158 analyzed using a phenol-hypochlorite assay (Weatherburn 1967). Lactate was
159 determined using a commercial kit (Megazyme Inc., Wicklow, Ireland). SCFA was
160 analyzed using a gas chromatograph (Shimadzu, Kyoto, Japan) equipped with an
161 ULBON HR-20M fused silica capillary column (0.53 mm i.d. × 30 m length, 3.0 μm
162 film; Shinwa, Kyoto, Japan). The procedures were as described in Watabe *et al.*
163 (2018).
164
165 **2.5 Microbial analysis**
166 Extraction of rumen DNA for microbial analysis was conducted using the
167 repeated bead-beating plus column method (Yu & Morrison 2004). Briefly, 250 μL

of ruminal fluid was mixed with glass beads and 1 mL of lysis buffer, and then physically processed to disrupt the microbial cells. The DNA was then precipitated with ethanol. The quality and quantity of DNA were assessed using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Thereafter, DNA was stored at −80 °C.

DNA was used for real-time PCR (qPCR) to quantify rumen representatives including total bacteria, *Butyrivibrio* group, *Ruminococcus flavefaciens, Fibrobacter succinogenes*, *Ruminococcus albus*, total bifidobacteria, genus *Prevotella*, *Ruminococcus bromii*, *Prevotella ruminicola, Selenomonas ruminantium, Ruminobacter amylophilus, Succinivibrio dextrinosolvens*, *Prevotella bryantii, Streptococcus bovis,* genus *Treponema*, *Treponema bryantii*, *Megasphaera elsdenii, Anaerovibrio lipolytica,* total methanogens and total protozoa.

Detailed information regarding qPCR such as primers, standards, PCR conditions, and calculations followed that of Ohene−Adjei *et al*. (2008) (for methanogen) and Koike *et al*. (2007) (for other microbes). For assaying *R. bromii*, the newly developed primers targeting the highly amylolytic group (namely, *R. bromii* C1 group) (forward, 5'- TGAGTGAAGTAGAGGCAGGC-3'; reverse, 5'-CCTATTAGAGTGCTCTTGCG-3'), were used under an optimized condition (annealing temperature, 59°C; extension time, 2min) (Koike *et al*. unpublished results). DNA was diluted to a final concentration of 10 μg/μL with 1 × TE (Tris−EDTA) buffer, and was then added to the reaction mixture (10 μL) consisting of 1 μL DNA, 0.1 μL each of forward / reverse primer, 5.0 of KAPA Taq PCR kit (Kapa Biosystems, Charlestown, MA, USA) and 3.8 μL of PCR-grade H_2O.

192

193 **2.6 Statistical analysis**

194 The experiment was carried out in a completely randomized design
195 arrangement to analyze the effect of the dietary starch source. All data were
196 analyzed using R (3.6.2 Version) software (R Core Team 2020) by a one-way
197 analysis of variance. Tukey's honestly significant difference test was then
198 conducted for multiple comparisons post hoc. The statistical model was as follows:

199 $Y_{ij} = \mu + T_i + \varepsilon_{ij}$, where Y_{ij} is the dependent variable in the treatment i for replication
200 j, μ is the overall mean, T_i is the treatment effect i and ε_{ij} is the random residual
201 error in the treatment i for replication j. Statistical significance was considered at
202 $P < 0.05$ and a trend was defined as $P < 0.10$.

203

204 **3. RESULTS**

205

206 **3.1 Growth performance and feed intake**

207 Table 3 shows the effect of dietary starch source on the growth performance
208 and feed intake of fattening cattle. The final body weight of fattening cattle fed
209 different starch sources did not differ. Weight gain and ADG were higher in cattle
210 fed PS compared to those fed GC. FCR was lower (more efficient) in cattle fed PS
211 than in those fed GC. Intake of DM, concentrate and roughage was not affected by
212 the dietary starch source.

213

214 **3.2 Blood parameters**

Blood lipid profiles including cholesterol, triglycerides, HDL, LDL and VLDL of cattle are presented in Table 4. Concentrations of cholesterol, triglycerides and VLDL did not significantly differ among the treatments. HDL tended to be different at day 74, showing a higher value in PS followed by CA and then GC. LDL was affected by the starch source, showing a consistently lower level in PS compared with CA. Level of LDL in CA was higher than that for GC on days 74, 147 and 206 and higher than for PS on days 147 and 206. PS diet decreased LDL levels in a feeding duration-dependent manner.

3.3 Rumen fermentation parameters

Rumen fermentation parameters in cattle fed the different starch sources are shown in Table 5. Ruminal pH did not differ throughout the experimental period. Cattle fed PS had consistently higher levels of total SCFA than those fed GC and CA. However, molar proportions of acetate, propionate and butyrate showed no difference among the three starch sources. Concentrations of NH_3–N and lactate were not significantly different among treatments.

3.4 Rumen microorganisms

Abundance of rumen microbes on days 74, 147 and 206 of the experiment is shown in Figure 1. Total bacterial abundance was not different among PS, GC and CA diets in all three sampling periods.

Butyrivibrio group and *R. bromii* as a member of amylolytic bacteria were consistently dominant throughout the experimental period, representing 11–27% and 11–42%, respectively. Particularly, *R. bromii* was detected at the highest

239 abundance in PS among the three treatments during the experimental period (with
240 only day 206 not showing significant differences). In the meantime, the
241 *Butyrivibrio* group showed the highest abundance in PS only in the sample on day
242 74. Although other amylolytics including *Prevotella*, bifidobacteria, *S.*
243 *ruminantium* and *R. amylophilus* showed inconsistent variation with treatment
244 and experimental period, all were minor members (4.70E−5 to 0.25%).

245 Three cellulolytic species, present at minor levels (0.0002−0.067%), did not
246 show apparent changes with treatment and experimental period. Other microbial
247 groups including lipolytics, intermediate-utilizers, and protozoa did not show
248 notable changes according to treatment and experimental period. Although the
249 relative abundance of methanogens was very high (17-25%), there was no
250 difference between treatments and periods.

251

252 ## 4. DISCUSSION

253

254 In discussing starch utilization in ruminants, the primary focus should be on
255 the ruminal degradation and fermentation of starch. As shown by the higher SCFA
256 levels in the rumen of cattle fed PS (Table 5), PS is considered to be more
257 extensively fermented in the rumen compared to the other two starch sources. It
258 is reasonable to suggest that the enhanced fermentation with PS could be
259 attributable to the high activity of amylolytic rumen bacteria (Figure 1), leading to
260 enhanced growth performance, as evidenced by ADG and FCR (Table 3), without
261 affecting blood lipid parameters partly indicating animal health status.

Starch digestion in the rumen can be influenced by chemical and physical characteristics of the starch source, such as the amylose/amylopectin ratio, granule size, and associated lipids and proteins (Svihus *et al.* 2005). In general, starch with a high amylose/amylopectin ratio, large granule size and high content of associated components is characterized by low degradability in the rumen. This is because amylose is less degradable than amylopectin, the associated components work as a surface barrier to enzymatic action, and the large granule size limits access by rumen microbes and their enzymes. Thus, it is important to take into account these factors in discussing the digestion properties of starch sources tested in the present study.

Stevnebo *et al.* (2009) characterized the ruminal starch digestion profile using barley cultivars with varying amylose content, and observed that barley with low amylose content showed higher degradability. However, this was not entirely related to starch per se, but was related to the higher degradability of NDF associated with the barley grain. In our study, the tested starch sources possess different NDF contents (lowest in PS < CA < GC, Table 1). This order corresponds well to that of SCFA level in the rumen of cattle fed the three different starch sources (PS > CA > GC, Table 5). In this regard, PS could be superior to the other starches with respect to rumen hydrolysis, as the low NDF content (Table 1) facilitates PS degradation and efficient fermentation. Svihus *et al.* (2005) emphasize the importance of starch structure in assessing starch degradability, i.e., the lipid complex and protein matrix associated with starch granules negatively affect starch degradation, since the digestion of such lipid and protein usually precedes starch digestion. According to the chemical properties of the three

starch sources, PS showed the lowest contents of crude protein and ether extract (Table 1), suggesting it has a simpler structure than GC and CA. In addition, PS had the smallest granule size (Table 1), which may facilitate access by rumen microbes and accelerate PS hydrolysis. Many reports have pointed out the decrease of starch digestion with the amylose/amylopectin ratio (Bednar *et al.* 2001; Saito *et al.* 2001; Abdel−Aal *et al.* 2002); however, the relationship appears to be more complex because of the different chemical and physical (structural) properties of the starch sources used in those studies. In this regard, PS used in the present study can be considered to be relatively pure and simple in chemical and structural properties compared to GC and CA, resulting in this starch source being more degradable and fermentable. Such an advantageous profile of PS could be derived from the industrial process of bromelain enzyme extraction.

Based on its fermentative nature (observed to be more degradable and fermentable), PS can be regarded as a risk factor for rumen acidosis, the rumen metabolic disorder occasionally observed under feeding of a high concentrate diet (Meyer & Bryant 2017). However, the ruminal pH was within the normal range, the ruminal lactate level remained low throughout the experiment (Table 5), and above all, no related symptoms were observed in any of the experimental cattle. Therefore, PS is considered to be a safe starch source when formulated as a 40% concentrate, as is generally done in Thailand. More detailed evaluation as to upper limit of PS supplementation is to be made in the future.

Rumen microbial results support the above scenario for PS, since amylolytic species, as represented by *R. bromii,* were observed at a higher abundance throughout the experimental period (Figure 1), possibly contributing to the higher

degradation and fermentation of PS compared to GC and CA. This could be the main reason for the higher SCFA production. Xia *et al.* (2015) showed the dominance of *Ruminococcaceae* (70–80%) in bacteria associated with barley grains, implying their contribution to starch hydrolysis. *R. bromii* is abundantly detected in the rumen of Japanese Black cattle, famous for its beef marbling under a high concentrate feeding system, suggesting the contribution of this species to rumen starch fermentation (Koike *et al.* unpublished results). The importance of *R. bromii* has been described for other ruminant livestock (Stewart & Bryant 1988; Herbeck & Bryant, 1974; Ze *et al.* 2013). These reports support our assertion that *R. bromii* plays a pivotal role in digestion of PS rich in amylose (35–36%) (Nakthong *et al.* 2017; Chen & Liu 1972).

Butyrivibrio is functionally versatile but possesses amylase activity and significantly contributes to starch hydrolysis, in particular amylopectin (Ramsay *et al.* 2006). *Butyrivibrio* of the amylolytic group increased with PS diet feeding at the initial period of the present study (Figure 1), which might partially explain the elevated hydrolysis and fermentation of the PS diet. Another major member, the genus *Prevotella,* which includes amylolytic species, did not respond to PS feeding as observed for minor amylolytic species (*S. ruminantium*, *R. amylophilus*, and others) (Figure 1).

In conclusion, pineapple stem starch feeding altered the rumen microbial community to support the growth and activity of the amylolytic *R. bromii* and *Butyrivibrio*. This promoted SCFA production in the rumen, primarily leading to improvements of body weight gain and feed conversion ratio. In this context, pineapple stem starch as a byproduct of the tropical fruit processing industry could

be applied as a feed ingredient for fattening cattle. More importantly, as this starch source did not influence feed intake and rumen pH, it is expected to be safe to use.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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Figure caption:

FIGURE 1 Effect of different starch source on rumen bacterial abundance of fattening cattle on 74th, 147th and 206th day (at 4h post feeding). The qualified species and groups are *Ruminococcus bromii*, *Butyrivibrio* group, Genus *Prevotella, Prevotella ruminicola*, *Prevotella bryantii*, *Succinivibrio dextionsovlvens, Selenomonas ruminantium, Ruminobacter amylophilus, Streptococcus bovis,* Bifidobacteria*, Fibrobacter succinogenes, Ruminococcus flavefaciens, Ruminococcus albus*, Genus *Treponema*, *Treponema bryantii, Megasphara elsdenii, Anaerovibrio lipolytica*, total methanogen and total bacteria. Means (n=12) with different letters are significantly different ($P < 0.05$). Error bar indicates SD. GC = ground corn; CA = ground cassava; PS = pineapple stem starch.