Novel Crabtree negative yeast from rumen fluids can improve rumen fermentation and milk quality

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Upgrading the nutritive value of rice straw (RS) is necessary to increase its contribution to enhancing meat and milk production. Present work verified whether novel Crabtree negative yeast inoculant could promote RS utilization, rumen fermentation, and milk quality in tropical crossbred lactating Holstein cows. The new stain of Crabtree negative yeasts (Pichia kudriavzevii KKU20 and Candida tropicalis KKU20) was isolated from the rumen of dairy cattle. This study used 6 multiparous crossbreds between Holstein Frisian × Zebu dairy cows in their mid-lactation period. Dairy cows were randomly allocated to three ensiled RS with various yeast stains including Saccharomyces cerevisiae, P. kudriavzevii KKU20, and C. tropicalis KKU20 according to a 3 × 3 replicated Latin square design. Crabtree-negative yeast (P. kudriavzevii and C. tropicalis) increased the apparent digestibility of dry matter by about 6.9% when compared with Crabtree-positive yeast (S. cerevisiae). Bacterial populations were highest with ensiled RS by C. tropicalis KKU20. Ensiled RS with Crabtree-negative yeasts were significantly increased with total volatile fatty acids, but they did not affect volatile fatty acid profiles. Milk protein percentage was highest at 35.6 g/kg when C. tropicalis was fed, and lowest when applied with S. cerevisiae and P. kudriavzevii KKU20 in ensiled RS at 34.5 and 34.1 g/kg, respectively. Thus, feeding ensiled RS with novel Crabtree negative yeast could improve RS digestion, rumen fermentation, and milk protein content in dairy cows.

Abbreviations
AA  Amino acids
ADF  Acid detergent fiber
ADFD  Acid detergent fiber digestibility
ADL  Acid detergent lignin
AFB1  Aflatoxin type B1
AIA  Acid-insoluble ash
BUN  Blood urea nitrogen
C2  Acetic acid
C3  Propionic acid
C4  Butyric acid
CEL  Cellulose
CP  Crude protein
CPD  Crude protein digestibility
CPI  Crude protein intake
DCP  Digestible crude protein
DIM  Day in milk
DM  Dry matter
DMD  Dry matter digestibility

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DMI  Dry matter intake  
ECM  Energy corrected milk  
EDTA  Ethylene diamine tetra-acetic acid  
EE  Ether extract  
FCM  Fat corrected milk  
GE  Gross energy  
HCELM  Hemicellulose  
HPLC  High-performance liquid chromatography  
IVOMD  In vitro organic matter digestibility  
KCF  Khon Kaen complete feed  
KKU  Khon Kaen University  
LA  Lactic acid  
ME  Metabolizable energy  
MPC  Microbial crude protein  
MUN  Milk urea nitrogen  
NDF  Neutral detergent fiber  
NDFD  Neutral detergent fiber digestibility  
NH₃-N  Ammonia nitrogen  
NRC  National Research Council  
OM  Organic matter  
OMD  Organic matter digestibility  
PDH  Pyruvate dehydrogenase  
RS  Rice straw  
SCC  Somatic cell count  
SNF  Solids-not-fat  
TDN  Total digestible nutrient  
TS  Total solids  
TVFA  Total volatile fatty acid  
VFA  Volatile fatty acid

As a result of rice production, million tons of rice straw (RS) are generated as a by-product; nevertheless, with low nutritional values. Various approaches are applied to enhance the nutritional value and improve its utilization in dairy cow's nutrition. The biological approaches are the most common with more harmless-treated RS. For several years, yeast has become an innovative biological model organism for enhancing animal efficiency and is the traditional practice for ruminant feed additives. Yeasts have been especially beneficial for single-cell protein creation and simply acceptable as their biomass has been used by ruminants in the form of fermented feed. In many studies, using yeast fermented with RS has been shown to enhance their nutritional value, silage quality, and nutrient digestibility. \textit{S. cerevisiae}, rapidly converts molasses and urea to provide biomass and greater nutrients from the whole-cell when added with oxygen (O₂) during the proliferation process. Wanapat et al. stated that \textit{S. cerevisiae} significantly increases crude protein (CP) in feedstuff via cell proliferation during the fermentation process and it provides essential amino acids, particularly lysine and methionine, for dairy cattle. Previous studies explained the benefit of live yeast in that it could provide a positive effect on feed utilization and performance production in the ruminants.

Although \textit{S. cerevisiae} has many benefits, several limitations have been reported, particularly that it produces low cell biomass. Under aerobic conditions, \textit{S. cerevisiae} exhibits alcoholic fermentation more than producing biomass. The “Crabtree-positive yeasts” are those that represent this characteristic. Under excessive glucose and even aerobic conditions, Van Urk et al. revealed that \textit{S. cerevisiae} had a limited proliferation capacity. Similarly, Wardrop et al. revealed that when cultivated with excessive glucose in a media solution, \textit{S. cerevisiae} provides 7 times lower biomass compared to other strains. This phenomenon restricts the chances of animals to receive highly nutritious from yeast biomass such as protein, essential amino acids, and vitamins. Consequently, it is important to extend the scope of research, and studying other yeast strains should be further improved. Crabtree-negative yeasts might be the most interesting option, as they have the special characteristic of limited fermentative products, biomass and carbon dioxide are the sole products under excessive glucose and aerobic conditions. Unlike Crabtree positive yeast, excessive glucose did not inhibit affected on ethanol production. The pyruvate has used another channel for converted to cytosolic-acetyl coA via acetaldehyde and acetate through pyruvate dehydrogenase (PDH) bypass channel into mitochondria. Consequently, high yeast biomass production more than Crabtree positive yeast may supply essential rumen fermentation factors and be a greatly nutritious feed supplement for ruminants.

Up to now, there is no research available information about the use of Crabtree negative yeast isolate from the rumen to improve RS. It was hypothesized that the novel Crabtree negative yeast inoculant could promote RS utilization, rumen fermentation, and milk quality in dairy cows. This research aimed to determine the effects of novel Crabtree-negative yeast (\textit{Pichia kudriavzevii} KKU20 and \textit{Candida tropicalis} KKU20) from rumen ensiled with RS and study their effect on feed digestion, ruminal fermentation, milk production, and milk composition of tropical crossbred lactating Holstein cows.
Results
Chemical composition of feeds. According to the experiment of De Deken the P. kudriavzevii KKU20 and C. tropicalis KKU20 was considered as Crabtree negative yeast, while S. cerevisiae as Crabtree positive yeast. In Table 1, the ensiled RS with different yeast species contained crude protein (CP) at 58.9 to 71.2 g/kg dry matter (DM) and 8.4 to 8.5 MJ/kg DM of metabolizable energy (ME), while the concentrate diet contained CP at 178.0 g/kg DM and 12.3 MJ/kg DM of ME. The neutral detergent fiber (NDF) content in ensiled RS with P. kudriavzevii KKU20 and C. tropicalis KKU20 were lower than in S. cerevisiae at 12.7% and 12.1%, respectively (Table 1). Fermentation quality of ensiled RS such as pH was ranged from 4.19 to 4.30, while lactic acid (LA), acetic acid (C2), butyric acid (C4), and ammonia nitrogen (NH3-N) were ranged from 19.8 to 21.9, 5.1 to 5.4, 0.81 to 0.82, and 1.8 to 2.0 g/kg DM, respectively.

Feed intake, nutrient intake, and nutrient apparent digestibility. The impacts of different yeast species ensiled RS on the effectiveness of feed utilization in dairy cattle is illustrated in Table 2. The yeast species did not change the RS intake, concentrate diet, and total intake (P > 0.05). Total intake ranged from 111.7 to 121.1 g/kg BW0.75. Organic matter (OM) and CP intake were 8.9 to 9.6 kg/day and 1.3 to 1.4 kg/day, respectively, which was not altered among treatments (P > 0.05). Crabtree-negative yeast (P. kudriavzevii KKU20 and C. tropicalis KKU20) increased the apparent digestibility of DM by about 6.9% when compared with Crabtree-positive yeast.

Table 1. Dietary ingredients and chemical composition of different yeast species in ensiled rice straw and concentrate diet. Premix = Vitamins and minerals; A: 10,000,000 IU; Vitamin E: 70,000 IU; Vitamin D: 1,600,000 IU; Fe: 50 g; Zn: 40 g; Mn: 40 g; Co: 0.1 g; Cu: 10 g; Se: 0.1 g; I: 0.5 g. EE ether extract, DM dry matter, CP crude protein, NDF neutral detergent fiber, ADF acid detergent fiber, ADL acid detergent lignin, HCEL hemicellulose, CEL cellulose, GE gross energy, ME metabolizable energy, LA lactic acid, C4 acetic acid, C2 butyric acid, NH3-N ammonia-N, Hemicellulose = NDF-ADF Cellulose = ADF-lignin Metabolizable energy calculated according to the equation described by Robinson et al. (2004). Crabtree Negative yeast as P. kudriavzevii = Pichia kudriavzevii and Candida tropicalis = C. tropicalis Crabtree Positive yeast as S. cerevisiae = Saccharomyces cerevisiae.
However, the data achieved in this study showed that apparent digestibility of OM (OMD), CP (CPD), NDF (NDFD), and acid detergent fiber (ADFD) were not altered among yeast species and ranged from 762.1 to 791.5, 752.0 to 791.5, 601.5 to 641.3, and 492.8 to 525.4 g/kg, respectively. Furthermore, the total digestible nutrients were the same among yeast species and ranged from 734.8 to 767.7 g/kg (P > 0.05).

Effect on rumen pH, NH₃-N, blood metabolites, and microbial communities. Table 3 and Fig. 1 illustrate the influence of ensiled RS with various yeast species fed to crossbred lactating dairy cows on ruminal pH, NH₃-N, blood urea nitrogen (BUN), and microbial communities. Rumen pH was not changed among yeast species, and the pH values were 6.4 to 6.8 (P > 0.05). Ruminal NH₃-N and BUN ranged from 16.5 to 22.1 mg/dL and 13.3 to 17.3 mg/dL, respectively (P > 0.05). The bacterial populations at both 0 h and 4 h after feeding and the mean value were highest (P < 0.05) with ensiled RS with C. tropicalis KKU20 by 9.9, 12.5, and 11.2 Log₁₀ cell/mL, respectively. However, the fungal zoospore and protozoa populations were not affected by any treatments (P > 0.05).

Effect on ruminal volatile fatty acid. The TVFA, C₂, C₃, C₄ proportions, and C₂/C₄ ratio are illustrated in Table 4 and Fig. 2. Ensiled RS with P. kudriavzevii KKU20 and C. tropicalis KKU20 were significantly increased with a total VFAs at 0 h (5.15 and 5.06%, respectively), and 4 h (5.07 and 8.83%, respectively) after feeding (P < 0.05) when compared with S. cerevisiae, whereas yeasts ensiled RS had no effect on the VFAs’ profile (P > 0.05). The mean value of C₂, C₃, and C₄ were 67.4, 22.3, and 10.3 mol/100 mol, respectively.

Effect on milk production, milk composition, and feed efficiency. The effects of ensiled RS with various yeast species on milk production, composition of milk, and feed efficiency in dairy cows are shown in Table 5 and Fig. 3. The yeast strains’ effects were not observed (P > 0.05) on actual milk yields (8.5 to 8.8 kg/h/day), 4.0% fat corrected milk (FCM) (7.6 to 8.3 kg/h/day), and energy corrected milk (ECM) (7.7 to 8.3 kg/h/day). The treatments did not alter the milk composition (P > 0.05); except for when the protein in the milk was...
Discussion

From NRC15, CP and ME requirements of our animal (BW 364 kg, milk yield 8.6 kg/day) increased by about 1,190 g/day and 60.9 MJ/day, respectively. The nutrient composition in feed, especially CP (provide 1,300–1,400 g CP/day) and ME (provide 79.05–84.14 MJ/day) values, were sufficient in our study for supporting dairy cows’ performance. Our study demonstrated that two yeasts were isolated from rumen fluids: \textit{P. kudriavzevii}-KKU20 and \textit{C. tropicalis}-KKU20. The name KKU refers to Khon Kaen University, where the strain was originally isolated, and the number “20” means the year of discovery, 2020. Presently, there are not many experiments has explored the impact of isolated yeasts on the mechanism of fermentation in the rumen. Intanoo et al.16 were isolated yeast from rumen fluids of three non-fistulated Thai-Holstein Friesian dairy cows and identified as \textit{Kluyveromyces marxianus} and \textit{Pichia kudriavzevii} for detoxifying aflatoxin B1 (AFB1) and it can apply further to use in animal feed. Correspondingly, Sirisan et al.17 found that there are three effective yeasts that could isolate from rumen fluids of Thai-Holstein Friesian dairy cattle including \textit{Pichia kudriavzevii}, \textit{Candida rugosa}, and \textit{Kodamaea ohmeri} which are used as preventing acidosis in dairy cattle diets. Although, these yeast species had previously been isolated and used for the ruminant animal18. However, the qualities of these strains on ensiled RS and animal production have not been studied. This is the first time that yeast has been used in animal feed for performance testing.

Ensiled RS with \textit{P. kudriavzevii} KKU20 and \textit{C. tropicalis} KKU20 (Crabtree-negative yeast) was established as having a low fiber content when compared with adding \textit{S. cerevisiae} (Crabtree-positive yeast). The low fiber content can be clarified by the yeast’s ability to release cellulase enzymes and digest fiber during the fermentation

Table 3. Effect of different yeast species in rice straw ensiled on ruminal microbial communities in crossbred lactating dairy cows. a,b Means in the same row with different superscript letters differ (P < 0.01, P < 0.05). Crabtree Negative yeast as \textit{P. kudriavzevii} = \textit{Pichia kudriavzevii} and \textit{Candida tropicalis} = \textit{C. tropicalis} Crabtree Positive yeast as \textit{S. cerevisiae} = \textit{Saccharomyce cerevisiae}.

| Items                      | \textit{S. cerevisiae} | \textit{P. kudriavzevii} KKU20 | \textit{C. tropicalis} KKU20 | SEM | P-value |
|----------------------------|------------------------|-------------------------------|-------------------------------|-----|---------|
| Rumen microbes, cells/mL   |                        |                               |                               |     |         |
| Bacteria, Log10 cell/mL    |                        |                               |                               |     |         |
| 0 h—after feeding          | 9.2\textsuperscript{a} | 9.4\textsuperscript{a}        | 9.9\textsuperscript{a}        | 0.28| p < 0.05|
| 4 h—after feeding          | 11.9\textsuperscript{b} | 12.2\textsuperscript{a}       | 12.5\textsuperscript{a}       | 0.22| p < 0.05|
| Mean                      | 10.5\textsuperscript{b} | 10.8\textsuperscript{a}       | 11.2\textsuperscript{a}       | 0.19| p < 0.01|
| Fungi zoospore, Log10 cell/mL |                      |                               |                               |     |         |
| 0 h—after feeding          | 7.8                    | 8.1                           | 8.1                           | 0.29| 0.31    |
| 4 h—after feeding          | 6.9                    | 6.9                           | 6.9                           | 0.33| 0.43    |
| Mean                      | 5.9                    | 5.7                           | 5.6                           | 0.24| 0.27    |
| Protozoa, Log10 cell/mL    |                        |                               |                               |     |         |
| 0 h—after feeding          | 4.3                    | 4.6                           | 4.6                           | 0.30| 0.38    |
| 4 h—after feeding          | 5.9                    | 6.2                           | 6.2                           | 0.27| 0.39    |
| Mean                      | 5.1                    | 5.4                           | 5.3                           | 0.17| 1.00    |

Figure 1. Comparison effects of ruminal Crabtree-Negative yeasts and Crabtree-Positive yeasts on rumen pH, ammonia nitrogen (NH$_3$-N) and blood urea nitrogen (BUN) of tropical crossbred lactating Holstein cows.
process. Suntara et al. confirmed that C. tropicalis KKU20 and P. kudriavzevii KKU20 were more capable of releasing cellulase enzymes than S. cerevisiae by about 0.7 to 6.8 times, respectively. The experiment on in vitro gas production of ensiled RS at 14 days with the P. kudriavzevii KKU20 could decrease the NDF content by about 6.7% when compared with S. cerevisiae. Ilmén et al. discovered yeast isolated from a plant named C. konsanensis species could excrete cellulase enzymes and digests fiber, and it is a new yeast strain that had not been reported previously. Similar to our study, C. tropicalis KKU20 and P. kudriavzevii KKU20 are great potential yeasts to improve feedstuffs and this study is the first report in ruminant nutrition feed research.

The fermentation quality of ensiled RS with different yeast species indicated that the silage was well preserved. The ensiled RS still maintained appropriate pH, high lactic acid content, and a low NH3-N level. Acceptable silage was defined by the pH value and the composition of their fermentation products. The pH is the main indicator for evaluating silage quality and our study showed ensiled RS still has a satisfactory score of about 4.1 to 4.3. The pH is highly related to LA content, which in this study showed a consistent range of about 19.8 – 22.1 g/kg DM. LA content in silage should range between 21 to 25 g/kg DM to be considered of high quality, according to Flieg's score. This is close to the high quality of silage. Our result showed LA content similar to an earlier study by Suntara et al. who revealed that about 20.53 to 26.14 g/kg DM of LA was produced when ensiled RS with C. tropicalis KKU20 and P. kudriavzevii KKU20 at 14 days. NH3-N concentration in ensiled RS within the range of 1.80 to 2.00 g/kg DM indicated the normal standards for estimating silage. These results are similar to those of Li et al., who collected information on various types of RS parameters and concluded that RS silage has a NH3-N concentration of approximately 1.61 to 2.36 g/kg DM. Other parameters such as C2 show great value for preserved silage within the range of 20 to 25 g/kg DM. After the fermentation process, the moisture content

| Table 4. Effect of different yeast species in rice straw ensiled on concentrations of total volatile fatty acid (VFAs) and their profiles in crossbred lactating dairy cows. *Means in the same row with different superscript letters differ (P < 0.01, P < 0.05). C2, Acetic acid, C3, Propionic acid, C4, Butyric acid. Crabtree Negative yeast as P. kudriavzevii = Pichia kudriavzevii and Candida tropicalis = C. tropicalis Crabtree Positive yeast as S. cerevisiae = Saccharomyces cerevisiae. |
|------------------|------------------|------------------|------------------|------------------|
| **Items**        | **S. cerevisiae**| **P. kudriavzevii KKU20** | **C. tropicalis KKU20** | **SEM** |
| **Total VFA, mmol/L** | 106.7a | 112.2b | 112.1a | 2.38 | P < 0.05 |
| **Volatile fatty acid profiles, mol/100 mol** | 64.9 | 65.8 | 65.9 | 0.86 | 0.35 |
| **C2** | 21.0 | 21.9 | 20.7 | 1.03 | 0.36 |
| **C3** | 22.6 | 24.1 | 23.2 | 1.13 | 0.32 |
| **C4** | 8.9 | 7.1 | 6.2 | 1.53 | 0.14 |
| **C2:C3** | 3.1 | 3.0 | 3.2 | 0.17 | 0.48 |
| **Figure 2. Comparison effects of ruminal Crabtree-Negative yeasts and Crabtree-Positive yeasts on the mean values ruminal total volatile fatty acid (TVFA), acetic acid (C2), propionic acid (C3), butyric acid (C4) and C2:C3 of tropical crossbred lactating Holstein cows.** |

![Figure 2. Comparison effects of ruminal Crabtree-Negative yeasts and Crabtree-Positive yeasts on the mean values ruminal total volatile fatty acid (TVFA), acetic acid (C2), propionic acid (C3), butyric acid (C4) and C2:C3 of tropical crossbred lactating Holstein cows.](image-url)
should range from 650 to 750 g/kg to be optimum24, which in our study showed an average of 722.1 g/kg. Hence, our study proposes that the nutrients in ensiled RS are still well preserved.

The Crabtree effect can be measured from the ratio, fermented glucose/respiration glucose, and positive if this ratio is > 1 and negative if the ratio is < 1. According to the experiment of De Deken25. This experiment indicated that Candida tropicalis is a Crabtree-negative yeast. Radecka et al.26 stated that Pichia kudriavzevii shows ability as a crabtree-negative yeast species. Another feature that shows the difference between Crabtree-positive and negative, with oxygen as the final electron acceptor, respiration is possible under aerobic conditions, but Crabtree-positive yeasts (such as S. cerevisiae) would inhibit PDH and produce ethanol instead of respiratory complete resulted in low cell growth27. In contrast, “Crabtree-negative” mean yeasts neglect fermentative products, and biomass and carbon dioxide are the primary products under aerobic conditions12. According to Suntara et al.6 reported that the rate of growth was revealed to be lower in S. cerevisiae (Crabtree-positive yeast) than in Candida tropicalis and Pichia kudriavzevii (Crabtree-negative yeast) (P < 0.01). All of the above reasons were ensured that both yeast strains including Candida tropicalis KKU20 and Pichia kudriavzevii KKU20 used in this experiment were Crabtree negative yeast.

Crabtree-negative or -positive yeast has no effect on the dry matter intake (DMI). Our results showed that the DMI (range from 2.6 to 2.8% BW) was similar to previous experiments, which is that feeding separate ensiled RS with a concentrate diet to dairy cows creates a DMI range from 2.5 to 3.2% BW28. Generally, the amount of RS that an animal intakes daily is limited to around 2.0% BW or less 2,29. Because RS is rich in polysaccharides and has a high lignin and silica content, and thus it limits the voluntary intake30. However, Aquino et al.31 reported that the amount of RS that ruminants can consume can be as high as 1.2% BW, which is similar to our result of 0.8–1.0% BW. The intake of OM, EE, NDF, and ADF was similar to previous studies of lactating crossbred dairy cows32,33. The CP intake (CPI) in this study was also similar with Wanapat et al.2, which used lactating crossbred dairy cows (50% Holstein Frisian × 50% Thai native cows) and BW around 365.5 kg, and the CPI was about 1.0 to 1.2 kg/day. Typically, the CP found in tropical forage plants is often relatively low34. Especially in RS (%CP) when using a roughage source it can have an effect on the animal’s yield adequacy35. Our study showed that ensiled RS with yeast could support protein from yeast to low-quality roughage as RS, and the enhanced intake of protein was high enough to meet the requirement of tropical lactation dairy cows.

Table 5. Effect of different yeast species in rice straw ensiled on milk yield, milk composition, feed efficiency and economic efficiency in crossbred lactating dairy cows. SCC somatic cell count, MUN milk urea nitrogen, DMI dry matter intake. DCP digestible crude protein. a,b Means in the same row with different superscript letters differ (P < 0.01, P < 0.05). Crabtree Negative yeast as P. kudriavzevii = Pichia kudriavzevii and Candida tropicalis = C. tropicalis Crabtree Positive yeast as S. cerevisiae = Saccharomyce cerevisiae. FCM fat corrected milk = 0.432 (kg of milk/day) + 16.23 (kg of fat), ECM energy-corrected milk = 7.20 × protein (kg/day) + 12.95 × fat (kg/day) + 0.327 × milk (kg/day).

| Items                        | S. cerevisiae | P. kudriavzevii KKU20 | C. tropicalis KKU20 | SEM | P-value |
|-----------------------------|---------------|-----------------------|---------------------|-----|---------|
| Actual milk yield, kg/h/day | 8.5           | 8.8                   | 8.6                 | 0.47| 0.09    |
| 4.0% FCM, kg/h/day          | 7.8           | 7.6                   | 8.3                 | 0.39| 0.11    |
| ECM, kg/h/day               | 7.8           | 7.7                   | 8.3                 | 0.30| 0.07    |
| Total solids, g/kg          | 122.7         | 121.2                 | 126.9               | 2.91| 0.09    |
| SCC, × 10⁵                  | 5.3           | 4.0                   | 6.6                 | 1.25| 0.10    |
| MUN, mg/dL                  | 12.9          | 13.1                  | 14.7                | 0.85| 0.06    |

Feed efficiency

| Milk/DMI                     | 0.90          | 0.87                  | 0.92                | 0.05| 0.62    |
| ECFM/DMI                     | 0.82          | 0.76                  | 0.89                | 0.06| 0.07    |
| DCP/Milk                     | 93.4          | 91.1                  | 97.3                | 4.02| 0.22    |

Figure 3. Comparison effects of ruminal Crabtree-Negative yeasts and Crabtree-Positive yeasts on milk protein and milk fat of tropical crossbred lactating Holstein cows.
The DMD was increased when ensiled RS with Crabtree negative yeast was offered to animals. This strain is outstanding in terms of high proliferation ability and its high yield of cellulase enzymes. The improved digestion may be due to the potential of how rumen microflora are promoted for better digestibility. Yeast is an important biological responder in rumen fermentation, live yeast cells improve microorganisms in rumen and stabilize pH in the rumen. Habeeb et al. stated that yeast could provide rumen with biological stimulants, which is necessary for microorganisms' growth in the rumen and yeast contributes to establishing microbiota and is why the digestibility was apparently enhanced. This is consistent with Wang et al., who found that Crabtree-negative yeast as C. tropicalis could increase digestion in the in vitro technique and that it generated 3.03% more gas production than did S. cerevisiae.

Crabtree-negative yeast did not change the apparent OMD, CPD, NDFD, and ADFD. The NDFD and ADFD are similar among Crabtree-negative and positive yeast (601.50 vs 650.05 g/kg DM and 492.8 vs 518.15 g/kg DM, respectively). Noticeable changes occurred after the silage process was complete, but when the animal intakes the feed, its digestibility was not altered. The reason for this is still not clear, but it is possible that yeast does not react directly on RS. Rather, digestion in the rumen occurred by the cooperation of microbes' synergy until the resulting values were not statistically different. This is similar to an experiment by Suntara et al., who compared the effect of Crabtree-negative and -positive yeast on ensiled RS on the in vitro gas and confirmed that in the rumen, there was no difference among yeast species in NDFD and ADFD (705.2 vs 703.6 and 464.8 vs 464.4 g/kg DM).

Ensilage RS with the P. kudriavzevii K20 and C. tropicalis K20 (Crabtree-negative yeast) could increase bacterial populations when compared to S. cerevisiae (Crabtree-positive yeast) by about 4.76%. The ruminal bacterial populations depend on sufficient nutrients or stimulants supply. Yeast is a great supply to stimulate bacteria because it is enriched in essential substances. Previous studies have confirmed that yeast could supply essential amino acids, vitamins, and minerals to increase the ruminal bacteria more than without yeast. The key explanation is that under aerobic conditions, Crabtree-negative yeast may proliferate more than Crabtree-positive yeast since the enzyme mechanism functions differently. Suntara et al. found that at 72 h of incubation time, P. kudriavzevii K20, C. tropicalis K20, and S. cerevisiae had growth by about 10.02, 9.6, and 8.87 Log cells/mL, respectively. The high amount of Crabtree-negative yeast creates a greater supply of essential nutrients to the rumen bacteria, thus the amount of rumen bacteria is increased in response to the Crabtree-negative yeast.

The ensiled RS with Crabtree-negative yeast has more effect on the total VFAs than with Crabtree-positive yeast by about 6.1% at the mean value. The high production of TVFAs in rumen fluids is related to the amount of ruminal bacteria. The great microbial population could enhance carbohydrate digestion and then the animal obtains the greater VFAs. This is similar to Castillo-González et al., who stated that the expansion of rumen microorganisms could increase the quantity of rumen VFAs. Certainly, a high bacterial population in our experiment was related to the Crabtree-negative yeast's effect. Nonetheless, the direct influence of the Crabtree-negative yeast on rumen bacterial populations was unclear and this hypothesis required further research to be conducted. Expanding the Crabtree-negative yeast population (during the fermentation process) may be more effective than expanding that of the Crabtree-positive yeast (S. cerevisiae). This suggests that animals have a greater chance of obtaining stimulants for activating rumen bacteria. In agreement with our results, Wang et al. compared the effect between Crabtree-negative yeast (C. tropicalis) and Crabtree-positive yeast (S. cerevisiae) for in vitro gas technique and found that the inclusion of 0.25 × 10⁷ of Crabtree-negative yeast could enhance the total VFAs by 7.7%. Suntara et al. reported that Crabtree-negative yeast (P. kudriavzevii K20) increased the total VFAs by 2.3% for in vitro gas study more than Crabtree-positive yeast.

The milk yield and milk composition of ensiled RS with Crabtree-negative yeast did not have any impact. Our study showed that the actual milk yields are about 8.5 to 8.8 kg/h/day, which are slightly lower than previous trials using early to mid-lactation cows (12.6 kg/h/day according to Supapong and Cherdthong). Our study showed that the actual milk yields are about 8.5 to 8.8 kg/h/day, which are slightly lower than previous trials using early to mid-lactation cows (12.6 kg/h/day according to Supapong and Cherdthong). The milk protein yields in the milk of the P. kudriavzevii KKU20 group were highest at 35.6 g/kg and lowest when applied with S. cerevisiae and P. kudriavzevii KKU20 in ensiled RS at 34.5 and 34.1 g/kg, respectively. Milk protein is associated with the feed degradation energy supply as VFAs and microbial crude protein (MCP) synthesis. High amounts of microorganisms in rumen can affect the MCP synthesis. This will be the supplied protein and amino acids effect between Crabtree-negative yeast (Crabtree-negative yeast) by about 4.76%. The ruminal bacterial populations depend on sufficient nutrients or stimulants supply. Yeast is a great supply to stimulate bacteria because it is enriched in essential substances. Previous studies have confirmed that yeast could supply essential amino acids, vitamins, and minerals to increase the ruminal bacteria more than without yeast.

Based on this study, the addition of a novel C. tropicalis K20 inoculant could help enhance RS quality and could promote DMD, the ruminal bacterial population, TVFAs, and the milk protein when compared with other groups. The ultimate objective has been achieved and new knowledge would play an important role in facilitating the implementation of rumen isolated yeast as a feed additive. However, there are certain drawbacks.
associated with the high-producing lactating cows influenced by *C. tropicalis* KKU20 treated RS, which requires further investigation.

**Methods**

The animals participating in this study have been certified by the Khon Kaen University Animal Ethics Committee (Record No. IACUC-KKU 38/62), based on the Ethics of Animal Experimentation of the National Research Council of Thailand. Our study confirmed that all methods were performed in accordance with the relevant guidelines and regulations.

**Isolation and identification procedure.** Two fistulated–crossbred Holstein Friesian steers, averaging 363.9 ± 55.80 kg (average milk yield 8.58 kg/day) and a mean age of 5 years. The milk yield reported was slightly higher than the previous studies, in which Holstein Frisian × Zebu cow’s milk yields were 2,897 kg/year or 8.05 kg/day51. Dairy cows were randomly allocated to three ensiled RS with various yeast species including *P. kudriavzevii* KKU20 and *C. tropicalis* KKU20 according to a 3 × 3 replicated Latin square design.

**Animals and experimental design.** This study used 6 multiparous crossbreds between Holstein Frisian × Zebu dairy cows in their mid-lactation period (165.5 ± 44.0 DIM) with an initial body weight of 363.9 ± 55.80 kg (average milk yield 8.58 kg/day) and a mean age of 5 years. The milk yield reported was slightly higher than the previous studies, in which Holstein Frisian × Zebu cow’s milk yields were 2,897 kg/year or 8.05 kg/day51. Dairy cows were randomly allocated to three ensiled RS with various yeast species including *S. cerevisiae*, *P. kudriavzevii* KKU20, and *C. tropicalis* KKU20 according to a 3 × 3 replicated Latin square design.

**Ensiling rice straw with yeast from rumen fluid.** The ruminal yeasts were obtained by isolating, screening, and identifying the rumen of crossbred Thai-Holstein Friesian dairy cattle8. The *P. kudriavzevii* KKU20 and *C. tropicalis* KKU20 (considered as Crabtree negative yeast25) were tested for their high-potential on in vitro study, which has an outstanding benefit for feed digestion and in vitro gas production8. The *S. cerevisiae* (considered as Crabtree positive yeast25) was obtained from the commercial baker’s yeast (PERFECT YEAST Co., Ltd, Ubon Rachathani, Thailand). In Fig. 4, isolated homogenous yeast suspension from the rumen (about 10⁶ cells/mL) was multiplied in media solution including 250 g molasses (Khon Kaen dairy cooperative Co., Ltd, Ubon Ratchathani, Thailand). In Fig. 4, isolated homogenous yeast suspension from the rumen (about 10^6 cells/mL) was multiplied in media solution including 250 g molasses (Khon Kaen dairy cooperative Co., Ltd, Ubon Ratchathani, Thailand). The isolated yeast has high potential to produce biomass and cellulase enzyme was selected to identification. DNA isolation from potential yeast was performed by boiling lysis buffer cells. The divergent D1/D2 domain of 26S rDNA was amplified with primers NL1-5′- GCA TAT CAA TAA GCG GAG 3′ and NL4-5′- GGT CCG TGT TTC AAG ACG G-3′. Amplification was performed in 100 μl of reaction mixture containing 100 ng of 2.5 U of Taq polymerase, genomic DNA, 40 mM of each primer, 20 mM of each dNTP, 1.5 mM MgCl2, and 10 mM Tris–HCl. The nucleotide sequences of the 26S rDNA D1/D2 domain were determined directly using PCR products, with slight modifications. Cycle sequencing of the D1/D2 domain was carried out with the forward primer NL1 (5′-GCA TAT CAA TAA GCG GAG GAA AAG-3′) and reverse primer, NL4 (5′-GGT CCG TGT TTC AAG ACG G-3′), using an ABI Prism™ BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Stafford, USA) according to the manufacturer’s instructions.

**Feeding and samples collection.** The feeding trial lasted for 63 days (21 days/period with 3 periods); dairy cows were held in independent pens and individually fed roughage and concentrate diets at 07:00 and 16:00. Ensiled RS offered ad libitum for all cows. The experimental diet was formulated by using the Khon Kaen Complete Feed (KCF) 2006 Program base on NRC13. The ingredients and nutrient composition of ensiled RS and concentrate diet were provided in Table 1. During the experiment, mineral blocks and fresh water were accessible. The experiment was performed over 3 periods with double squares. The period lasted for 21 days, the first 14 days for treatment adjustments and the last 7 days for sample intake and collection assessment.

At the time of the feeding trial, orts were obtained and weights were collected every day, and the feeding rate was adjusted daily to yield orts between 50 to 100 kg/kg of intake. Individual voluntary feed determined consumption difference between the feed offered and orts. Around 5 g/kg of the overall fresh fecal samples were split into two parts; the first part of each day for DM analysis and the second part were pooled at the end of each period. The pooled fecal samples (500 g) were stored at −20 °C until analysis. At 60 °C, composite samples were dried,
pressed through a steel filter of 1 mm for grinding (WILEY MILL, Arthur H. Thomas Co., Ltd., Philadelphia, PA, USA), and then analyzed for DM (ID 967.03), ash (ID 492.05), ether extract (EE; ID 455.08), CP (ID 984.13) content53, NDF, ADF54, and acid-insoluble ash (AIA)55. Body weights were measured every period. The calculation of ME was based on the equation defined by Wachirapakorn et al.32,56:

\[
\text{ME (MJ/kg DM)} = 0.82 \times [2.4 \times \text{CP} + 3.9 \times \text{EE} \times 1.8 \times \text{the rest of the OM}] \times \text{in vitro organic matter digestibility (IVOMD)}
\]

where CP, EE, and OM are in g per kg of DM and IVOMD with the mean values received from our recent in vitro study with mean values of 682.5 g/kg DM.

The 10 g of fresh silage was blended with 90 mL of sterilized water and stored at 4 °C57,58. The pH of the ensiled RS was measured by a pH meter using cold-water extracts (HANNA HI-8424 Portable pH/ORP Meter, Woonsocket, USA) according to Pholsen et al.59,60. Silage fluid subsamples were centrifuged for 15 min at 6,000 rpm and the liquid above the solid residue was filtered using a 0.45-micron syringe filter. High-performance liquid chromatography (HPLC) devices (SHIMADZU LC-20A, Shimadzu Industrial Systems Co., Ltd, Kyoto, Japan) were used to conduct LA, C2, C3, and C4 analyses61. The NH3-N concentration was calculated according to the Kjeldahl process53. Jugular blood and rumen fluid samples were obtained at 0 and 4 h after feeding on the last day of each period. A blood sample (approximately 10 mL) was obtained in tubes containing 12 mg of ethylene diamine tetra-acetic acid (EDTA) from the jugular vein. The plasma was isolated by centrifugation for 10 min at 500×g and preserved at −20 °C until BUN analysis, according to Abdallah et al.62. Approximately 200 mL of rumen fluid was collected from the rumen by a stomach tube connected to a vacuum pump. Rumen fluid samples were then filtered through 4 cheesecloth layers. A fluid sample containing 5 mL of 1 mol/L of H2SO4 applied to 45 mL of rumen fluid was put into the bottle. The rumen fluid mixture was centrifuged for 15 min at 6000×g and used for analyzing the NH3-N53 and VFA (gas chromatography, MODEL HP6890-HEWLETT, NY, USA)63. Ruminal bacteria, protozoa, and fungal zoospores were numbered under a hemocytometer using the direct counting method64.

During the last 7 days of each experimental period, milk samples were taken according to the yield for morning and afternoon milking, preserved with 2-bromo-2 nitropropane-1, 3-dial, and stored at 4 °C until analysis by using Milko-Scan (FOSS ELECTRIC, Hillerod, Denmark) for fat, true protein, lactose, total solids (TS), and solids-not-fat (SNF) content. Milk urea nitrogen (MUN) was estimated by the diacetyl monoxime method using UV/Vis-spectrophotometer (PG Instruments Ltd., London, UK) according to Aguilar et al.65. Fat, protein, lactose, TS, and SNF concentrations were measured as weighted media depending on morning and afternoon milk yields per each test day by infrared methods using Milko-Scan 33.Yields of 4.0% FCM were calculated according to Rafferty et al.66:

\[
\text{FCM fat corrected milk} = 0.432 \text{ (kg of milk/day)} + 16.23 \text{ (kg of fat)}
\]

while yields of ECM were calculated as described by Krause and Combs87:

\[
\text{ECM energy-corrected milk} = 7.20 \times \text{protein (kg/day)} + 12.95 \times \text{fat (kg/day)} + 0.327 \times \text{milk (kg/day)}
\]

For each cow and period, feed conversion efficiencies were determined by dividing the average yield of actual milk and ECM by the respective DMI and digestible protein per yield of actual milk85.
Statistical analysis. All data from the experiment were analyzed according to a 3 × 3 replicated Latin square design using the GLM procedure\(^\text{48}\) according to the model:

\[
Y_{ijkl} = \mu + S_i + M(l) + A_j + P_k + e_{ijkl}
\]

where \(Y_{ijkl}\) observation from cow \(j\), receiving ensiled RS \(i\), in period \(k\); \(\mu\), the overall of mean, \(S_i\), the effect of square \((l = 1, 2)\); \(M(l)\), effect of yeast species in RS silage \((l = 1, 2, 3)\); \(A_j\), the effect of cows \((j = 1, 2, 3, 4, 5, 6)\); \(P_k\), the effect of period \((k = 1, 2, 3)\); and \(e_{ijkl}\), the residual effect. Significant differences between individual means were evaluated using Duncan’s multiple comparison tests when a significant \((P < 0.05)\) effect was detected\(^\text{49}\). Standard errors of means were calculated from the residual mean squares in the analysis of variance.

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Author contributions
C.S., A.C., and S.U.: Investigation, Methodology; C.S., A.C.: Data curation, Formal analysis, Software, and Project administration, Conceptualization, Methodology, and Project administration, Funding acquisition; C.S., A.C., and S.U.: Resources, Supervision, Validation; Visualization; C.S.: Roles/Writing—original draft; C.S., A.C., M.W., and P.C.: Writing—review & editing. All authors have read and agreed to the published version of the manuscript.

Competing interests
The authors declare no competing interests.

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